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ACTIVATION INDUCED CELL DEATH DURING T CELL DEVELOPMENT AND IN T CELL HYBRIDOMAS

BY ©
YUFANG SHI

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY.**

IN

MEDICAL SCIENCES (IMMUNOLOGY)

Edmonton, Alberta
Fall, 1992

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **ACTIVATION INDUCED CELL DEATH DURING T CELL DEVELOPMENT AND IN T CELL HYBRIDOMAS** by **YUFANG SHI** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **MEDICAL SCIENCES (IMMUNOLOGY)**.

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To the Memory of My Father

Qingyun Shi

ABSTRACT

Thymic negative selection of developing T cells is probably central to self-nonself discrimination. Very little, however, is known about its mechanism. The hypothesis being examined in this thesis is that T cells enter a suicide pathway (apoptosis) after being activated by antigens in association with MHC during maturation in the thymus. To test this hypothesis, thymocytes were activated non-specifically by *in vivo* administration of antibodies to the CD3-T cell receptor complex. This treatment causes cortical immature thymocytes to undergo apoptosis, as characterized by fragmentation of genomic DNA into a nucleosome-sized ladder, nuclear chromatin condensation and membrane blebbing.

An *in vitro* model with T cell hybridomas can mimic the *in vivo* phenomenon. Upon activation, T cell hybridomas also showed chromatin condensation, cell membrane blebbing and nuclear DNA fragmentation. These changes were dependent on newly synthesized macromolecules, since death was significantly reduced by inhibition of RNA or protein synthesis.

A nuclease inhibitor, aurintricarboxylic acid (ATA), was found to inhibit cell death. It also inhibited Ca^{++}-dependent nuclease induced DNA fragmentation in isolated normal nuclei. These and the observation that ATA acts at or near the time of DNA fragmentation from kinetic studies suggest that DNA fragmentation is the initiator of activation-induced cell death in T cell hybridomas. The ability of ATA to inhibit activation-induced apoptosis in thymocytes *in vivo* supports a similar role for DNA fragmentation in this setting.

An immunosuppressive drug, cyclosporin A, which can interfere with negative selection in the thymus and which induces autoimmunity upon withdrawal, blocked cell death both *in vivo* and *in vitro,* providing a link between activation-induced cell death and

negative selection. Further, TGF-β1, TGF-β2 and synthetic oligonucleotides antisense to *c-myc* blocked cell death, providing important clues toward our understanding of the mechanism of activation-induced apoptosis.

We conclude that the cell death induced by activation of the cortical thymocytes *in vivo* and T cell hybridoma *in vitro* is a process involving apoptosis. Further studies of the *in vitro* hybridoma system, with its capacity for monoclonal recognition, will provide more information about the mechanism of activation-induced apoptosis as it relates to negative selection in the thymus.

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ABBREVIATIONS

ACID	Activation-induced cell death
Ab	Antibody
Ag	Antigen
ANOVA	Analysis of variance
APC	Antigen presenting cells
AS	Antisense
ATA	Aurintricarboxylic acid
AtD	Actinomycin D
B cell	Bone marrow derived lymphocyte
BM	Bone marrow
CD	Cluster of differentiation
CD3	T cell receptor associated molecules
CD4, CD8	T cell accessory molecules
CHX	cycloheximide
CMI	Cell mediated immunity
ConA	Concanavalin A
CPM	Counts per minute
CsA	Cyclosporin A
DAG	1,2-diacylglycerol
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate conjugate
H-2	Mouse major histocompatibility complex
HLA	Human major histocompatibility complex

HSA	Heat stable antigen
Ig	Immunoglobulin
i.p.	Intraperitoneal
IP3	Inositol 1,4,5-triphosphate
i.v.	Intravenous
LK	Lymphokine
LN	Lymph node
MHC	Major histocompatibility complex
MMTV	Mouse mammary tumor virus
MTT	Tetrazolium
MLN	Mesenteric lymph node
Mls	Minor lymphocyte stimulatory antigen
NHIg	Normal hamster immunoglobulin
P	Probability
PBS	Phosphate buffered saline
PKC	Protein kinase C
PLC	Phospholipase C
PMA	4-β-phorbol 12-myristate, 13-acetate
SCID	Severe combined immunodeficiency
SE	Standard error of the mean
SEB	Staphalococcal enterotxin B
T cell	Thymus derived lymphocyte
TCR	T cell antigen receptor
TH1, TH2	T helper cell subsets
Thy-1	Mouse T cell specific surface marker
TGF	Transforming growth factor
TNF	Tumor necrosis factor

V	Variable region

I. INTRODUCTION

Self-nonself discrimination is a hallmark of the immune system. Most immune responses, regardless of their effector functions and mechanisms, depend upon regulation by helper T lymphocytes. T lymphocytes are continuously derived from the thymus throughout the life of vertebrates; therefore, the thymus has been a focus of studies of immunological self-nonself discrimination, or immune tolerance. It has been established in recent years that during development in the thymus, potentially self-reactive T cells are purged, or negatively selected, from becoming mature lymphocytes. However, the mechanism by which negative selection occurs is undefined. The working hypothesis of this thesis is that autoreactive T cells enter a suicide pathway after being activated by self antigens at some immature stages during development, while mature T cells become resistant to this activation-induced cell death.

A. General Review

The Immune System

The vertebrate immune system distinguishes substances of self origin from those of foreign origin. Invading foreign materials are recognized as antigens and elicit immune responses that destroy or remove them specifically. After being in contact with an antigen, the immune system is prepared to make a faster and stronger reaction if exposed to that antigen a second time. The capability of the immune system for specific recognition and memory of antigens is attributed to T lymphocytes derived from the thymus and B lymphocytes derived from the bone marrow (or the avian bursa of Fabricius), with the help of antigen nonspecific, bone marrow derived accessory cells.

Two general types of immune reaction occur: humoral immunity and cellular immunity. Humoral immunity is mediated by B cell secreted antibodies. Antibodies bear specific sites for antigen recognition, and are responsible for neutralization of toxins, opsonization for phagocytosis, antibody-dependent cell-mediated cytotoxicity, antibody-mediated mast cell degranulation and complement cascade activation.

Cellular immunity is mediated by T cells. T cells recognize antigens when they are present on the surface of antigen-presenting cells in association with the major histocompatibility (MHC) antigens. One type of cell mediated immunity is through direct cell-cell contact. This interaction can cause death of transformed cells, pathogen-infected cells or cells of a foreign graft. Another type of cell-mediated immunity is delayed-type hypersensitivity, during which antigens are destroyed by macrophages under the influence of T cell products known as lymphokines. T cell-secreted lymphokines are also responsible for the regulation of both humoral and cellular immune responses. Depending upon the physiological status of an individual, different antigens initiate different patterns of lymphokine production, which in turn determine the types of immune responses.

Organization of the Immune System

Cells responsible for immune responses are constantly replenished by multipotent hematopoietic stem cells from bone marrow throughout the life of an organism. Proliferation and differentiation of hematopoietic cells are tightly regulated by the bone marrow microenvironment (Dorshkind, 1990). There are two lineages from the multipotent stem cells: lymphoid and myeloid. The myeloid lineage generates red blood cells, platelets, neutrophils eosinophils, and the accessory cells for immune functions. The lymphoid lineage gives rise to B and T cells (Golub and Green, 1991). In birds, the bursa of Fabricius is the site for B cell differentiation (Glick et al, 1956), and in mammals, bone marrow or the Peyer's patches are the avian bursal equivalent (Reynaud, et al., 1991). T cell precursors undergo maturational differentiation processes in the thymus to become functional mature T cells. These lymphoid precursor differentiation sites are collectively called primary lymphoid organs. After differentiating in the primary lymphoid organs, mature functional lymphocytes migrate to spleen, lymph nodes or other lymphoid tissue in the body. These peripheral lymphoid organs are called secondary lymphoid organs.

Antigen Receptors and Clonal Selection

An important process occurring during the differentiation of both B and T lymphocytes is the somatic rearrangement of genes for one antigen receptor from many possible genomic rearrangements (Brack and Tonegawa, 1977; Hedrick et al., 1984). The antigen receptor determines the antigen specificity of a cell. Each T or B cell surface has many identical receptors, with the same antigen specificity. Cells having the same type of receptor constitute a clone. Each receptor can only recognize one antigenic epitope. An individual organism has many clones with the capacity as a whole of recognizing virtually any foreign antigen and an invading antigen will be recognized by one or a few particular clones. After recognizing an antigen, each antigen-specific lymphocyte proliferates and differentiates to become a clone of antigen specific, functional cells. This process has been referred to as clonal selection (Burnet, 1959).

T Cell Surface Markers

Although T and B lymphocytes are indistinguishable morphologically, some of their surface molecules can be used to differentiate among them. The first murine T cell marker defined was the Thy-1 antigen, a 24-kd glycoprotein (Raff, 1971). A more conclusive marker for murine and human T cells is the CD3 (or T3) complex, a group of peptides associated with T cell antigen receptors (TCR) expressed on the cell membrane (Weiss and Imboden, 1987). The development of monoclonal antibodies against lymphocyte surface markers has immensely advanced our knowledge of lymphocytes. Lymphocyte surface molecules are named under the CD (cluster of differentiation) nomenclature system, with many identified to date. CD4 and CD8 are two of the most widely used CD molecules in T cell biology. Mature T cells can be divided into two populations on the basis of these CD molecules. About two-thirds of them express CD4 but not CD8, and one-third CD8 not CD4 (Shevach, 1989).

MHC

There are two classes of MHC, class I and class II. Class I is expressed on most nucleated cells [one known exception is human villous trophoblasts (Faulk et al., 1977)]. Class II, with a more restricted tissue distribution, is present on antigen-presenting cells such as activated B cells, macrophage/monocytes, dendritic cells, activated human T cells and epithelial cells of the thymus. The MHC genes are located on chromosome 17 in mouse (H-2) and chromosome 6 in human (HLA). The highly polymorphic MHC molecules are heterodimeric glycoprotein consisting of two chains. The extracellular parts of the two chains of the class II molecule are each composed of a polymorphic domain and a constant domain. The class I molecule consists of a transmembrane heavy chain with three extracellular domains noncovalently associated with β_2-microglobulin. The polymorphism of class I molecules resides in the two membrane distal domains of the heavy chain. The x-ray crystal structure of human class I HLA-A2 shows that the two polymorphic domains fold into a platform of eight antiparallel β-strands topped by two α-helices (Bjorkman et al., 1987). The two α-helices run parallel to each other and form the wall of a cleft that contains proteolytically processed peptide antigens (Madden et al., 1991; Fremont et al., 1992). A proposed model for class II is similar to that of class I (Brown et al., 1988).

MHC Restriction

Unlike B cells, T cells do not recognize antigen alone. Instead, they only recognize antigens that have been processed and presented on the surface of antigen presenting cells (APC) in association with MHC antigens. In addition, APC and T cells must originate from the same or a syngeneic individual. This requirement for histocompatibility is called MHC restriction (Zinkernagel and Doherty, 1974). There are two types of T cells: MHC class I restricted CD8+ T cells and MHC class II restricted CD4+ T cells. In general class I proteins present antigens derived primarily from the cytosol. Class II proteins present antigens derived from lysosomally degraded endocytosed external proteins (Carbone and Bevan, 1989).

After stimulation by antigen, CD4+ T cells develop into helper T cells, helping B cells to produce antibodies, or helping CD8+ cells to expand clonally and develop into cytotoxic T cells. According to the pattern of cytokines they produce, CD4+ cells can be further divided into different subsets (Mosmann et al., 1986). The functions of CD4+ and CD8+ cells are not mutually exclusive; both may produce lymphokines and become cytotoxic.

T Cell Activation

The interaction of T cells with antigen and MHC complex, with antibodies against the T cell receptor complex, or with mitogenic lectins generates second messengers inside T cells, which further activate T cells. It has been demonstrated that T cell receptor occupancy leads to activation of phospholipase C (PLC) (Manger et al., 1987), which cleaves membrane phosphotidalinositol 4,5-biphosphate, giving a transient increase in the production of 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). The former causes translocation of protein kinase C (PKC), a serine/threonine kinase, from the cytosol to the membrane-associated compartment. IP3 increases the intracellular calcium concentration. Studies using DAG analogue, PMA, and Ca^{++} ionophore have demonstrated that both PKC and Ca^{++} influx are required for the activation of T cells (Truneh et al., 1985). It has not been demonstrated if activation of PLC in T cells require guanine nucleotide binding protein. However, it is clear that the ligation of T cell antigen receptor leads to the activation of tyrosine kinases (Klausner and Samelson, 1991). It has been proposed that these kinases might phosphorylate PLC and activate it. It has been shown that tyrosine kinase specific inhibitors, genestin and herbimycin, could inhibit TCR ligation induced PI turnover (Klausner and Samelson, 1991; Mustelin et al., 1990).

The generation of the secondary messengers activates many lymphokine genes and protooncogenes required for cell division. The increased messenger RNA transcripts or translation products can be regulated either by transcriptional or by post-transcriptional regulation. Some genes are activated within the first 15 minutes after stimulation and some within the first hour, while others require days (Schwartz, 1990).

Tolerance

The immune system is designed to recognize external invaders and internal malignant cells by clonally distributed, diversified antigen receptors on both T and B lymphocytes. Antigen receptors of T and B cells are generated in their diversity throughout the life of an individual by random rearrangement of DNA segments encoding the variable portion of the receptors and by random pairing of these variable protein chains. There are at least 10^{11} possible different immunological receptors; thus, whatever the antigen is, it is highly likely that there will be a complementary receptor to recognize it (Koshland, 1990). A problem with such diversified specificity is that the immune system must distinguish foreign carbohydrates, nucleic acids, proteins and other antigens from those of self. When it is working well, the immune system never responds to self, but responds unerringly to nonself. When it is not working well, autoimmune disasters occur.

The receptors for both self and nonself are generated during the development of immune cells, and self and nonself discrimination, or self-tolerance, has to be learned somatically. As early as the 1890s it was realized that even nonpathological substances such as serum proteins and red blood cells could stimulate antibody production. Further tests with self serum or other self proteins showed negative results, leading Ehrlich to formulate the concept of *horror autotoxicus,* in which an individual is unable to mount a destructive response to self-constituents. However, Metchnikoff's experiments reported that cells from many organs, such as spermatozoa, induced specific antibodies to the immunizing cells. It was soon discovered in clinical investigations that autoreactivity was indeed involved in some eye diseases (Reviewed by Silverstein, 1989), and is apparent now that autoimmunity is the cause of many clinical diseases.

Studies on how the immune system learns tolerance somatically constitute one of the most active areas in current medical and biological research. Our current understanding is that the immune cells bearing specificity to self are rendered tolerant by three mechanisms. One mechanism is called clonal deletion, which involves the elimination of those cells that would otherwise initiate antiself responses. The second mechanism is clonal anergy, involving the inactivation of these autoreactive cells. The last mechanism is through the generation of suppressor cells that suppress the activity of autoreactive cells. This thesis is designed to study the mechanism of activation-induced cell death during T cell development and its relationship to clonal deletion of autoreactive cells.

B. THE THYMUS

The thymus is derived from the third endodermal pouch and the third ectodermal cleft of the gill branchial apparatus during embryological development. It is formed by cells from the ectodermal third cleft which proliferate intensively to cover the endodermal third pouch. Thus, the thymus epithelial cells are composed of the endoderm-derived medulla and ectoderm-derived cortex (Cordier and Haumont, 1980). The thymus epithelial cells, together with bone marrow derived dendritic cells, provide the maturational environment for T cells.

The development of T cells in the thymus has attracted tremendous interest among scientists in recent years, for three reasons. First, the differentiation of lymphocytes in the thymus is a continuous process during the life of an individual, which offers an unique model system to study tissue and stage-specific gene regulation during mammalian development. Second, intrathymic events, such as T cell receptor gene rearrangement, and interaction of developing thymocytes with other cells in the thymus, are known to be the key points in the development of a self-tolerant functional T cell repertoire. Third, the same T cell receptor on the surface of thymocytes at different developmental stages delivers different signals, leading to different selection processes, providing an excellent model for studying differential signal transduction.

The importance of the thymus in the development of a functional immune system was appreciated more than three decades ago when it was recognized as producing and exporting immunocompetent mature T lymphocytes to the peripheral lymphoid organs (Miller, 1961 and Reviewed in Stutman et al., 1969). There are two lines of evidence. First, neonatally thymectomized mice have no or very few T cells in the peripheral lymphoid organs; likewise, T cells fail to develop in individuals congenitally lacking the thymus, for example, nude mice or humans with DiGeorge syndrome. More directly, *in situ* labeled thymocytes have been observed to leave the thymus for the peripheral lymphoid tissues (Scollay and Shortman, 1985). Sprent (1977) showed that about 1-2 x 10^6 cells are released from the thymus to the periphery per day in young mice. The thymus is also responsible for shaping the T cell repertoire (Zinkernagel and Doherty, 1979). Egerton et al. (1990) reported that the rate of mature thymocytes being exported from the thymus is only 3% and no cell division is observed during migration from the cortex to the medulla.

After arriving at the thymus, thymic precursors derived from bone marrow are mitotically active, then give rise to nondividing small lymphocytes with a life span of no more than 3-4 days. This has been recently confirmed by Huesmann et al. (1991) with T cell receptor transgenic mice (discussion follows). By using continuous ^{3}H-thymidine labeling, it was shown that most of the cortical thymocytes become labeled within three days, while medullary cells become labeled much more slowly. Taking into account the cell number in both compartments, many fewer cells became medullary cells than became cortical cells (Scollay and Shortman, 1985), indicating that many cells died in the cortex. McPhee et al. (1979) labelled mice simultaneously with the efficiently reutilizable ^{3}H-thymidine and a poorly reutilizable analogue, ^{125}I-deoxyuridine, and found that there was a fast, thymus specific loss of ^{125}I-deoxyuridine, indicating that a large number of thymocytes die within the thymus. However, Quackenbush and Shields (1988) found that lower reutilization of iododeoxyuridine was due to deiodonation after comparing the reutilization of different isotopes labeled thymidine and iododeoxyuridine, raised suspicion about the reliability of early data about rapid cell death in normal thymus. When thymocytes labelled continuously with ^{3}H-thymidine were analyzed with the combination of several defined T cell markers, it was found that only 3% of immature thymocytes become mature cells, clearly indicating that there was extensive intrathymic cell death of immature thymocytes (Egerton et al., 1990). However, it has not been be possible to find DNA fragmentation in freshly isolated thymocytes (Hugo et al., 1990; Smith et al., 1989) and cell death in normal thymocyte sections (Reinherz and Schlossman, 1980). This could be because the cell death process is fast and the dead cells are quickly taken up by macrophages (Savill et al., 1990). Indeed cell death can be detected when the phagocytic system was overloaded by treating thymus with cell death inducing agents (Smith et al., 1989), or thymocytes are cultured in the absence of macrophages (MacDonald and Lees, 1990).

The cells migrating from the thymus to peripheral lymphoid tissues bear the phenotypes of the medullary thymocytes (Scollay et al., 1978). The lineage relationship between the rapidly dividing cortical population and slowly turning-over medullary thymocytes was established (Reviewed by Scollay, 1991) by injecting purified thymocyte subpopulations. By staining with different antibodies directed to cell surface molecules such as CD4, CD8 and T cell receptors, thymocytes can be divided into several developmental stages (Sprent and Webb, 1987). Considerable *in vivo* information about different populations has been obtained by using these markers to separate and analyze different thymocyte populations. Thymocytes first express both CD4 and CD8 at low levels, then at high levels and subsequently develop into $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ (von Boehmer, 1988). At the early double positive ($CD4^{+}CD8^{+}$) stage, thymocytes randomly rearrange and express T cell antigen receptor on the cell surface in association with a group of proteins called CD3.

A current understanding of intrathymic developmental pathways for T cells is summarized in Fig. 1 (adopted from Nikolic-Zugic, 1991). T cell precursors are derived from multipotential hemapoietic stem cells. During murine development, fetal liver derived precursors migrate to the thymus on day 11 (Owen and Ritter, 1969). Adult precursors originate from bone marrow. The precursors are $CD44^{+}$ and Thy-1^{lo}, and are negative for TCR, CD4, CD8 and IL-2 receptors (IL-2R). These cells enter the thymus either through the thymic corticomedullary junction or the cortical subcapsule under the influence of the thymic epithelia-derived chemoattractants such as β2-microglobulin (Zijlstra et al., 1990). Soon after migrating from the bone marrow to the thymus, T cell precursors express high levels of heat stable antigen (HSA^{+++}) and remain $CD4^{-}CD8^{-}CD3^{-}$(Nikolic-Zugic, 1991). These so-called triple negative cells account for 1-2% of the total thymocytes and are mainly concentrated under the capsule in the outer cortex (Sprent, 1989). These cells then start to proliferate, transiently expressing IL-2R and cytoplasmic mRNA of the β-chain of TCR. Thereafter, the expression of IL-2R is downregulated followed by transcription of α-chain of TCR and low-level expression of TCR on the surface. It has been suggested that before the expression of TCR, there are

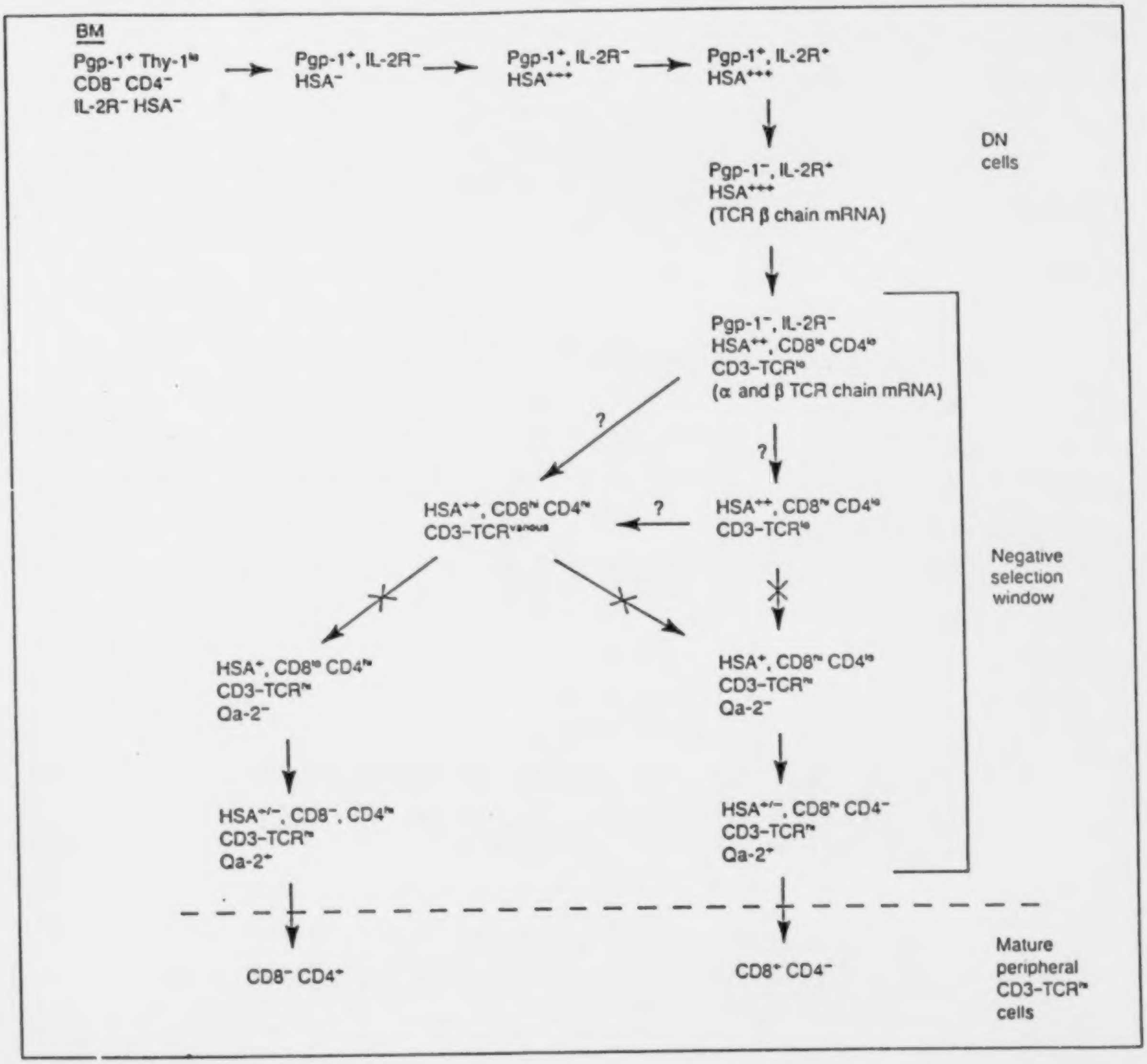

BM
Pgp-1$^+$ Thy-1^{lo} CD8$^-$ CD4$^-$ IL-2R$^-$ HSA$^-$
Pgp-1$^+$, IL-2R$^-$ HSA$^-$
Pgp-1$^+$, IL-2R$^-$ HSA^{+++}
Pgp-1$^+$, IL-2R$^+$ HSA^{+++}
DN cells
Pgp-1$^-$, IL-2R$^+$ HSA^{+++} (TCR β chain mRNA)
Pgp-1$^-$, IL-2R$^-$ HSA^{++}, CD8^{lo} CD4^{lo} CD3-TCR^{lo} (α and β TCR chain mRNA)
?
?
HSA^{++}, CD8^{hi} CD4^{hi} CD3-TCR^{various}
?
HSA^{++}, CD8^{hi} CD4^{lo} CD3-TCR^{lo}
Negative selection window
HSA$^+$, CD8^{lo} CD4^{hi} CD3-TCR^{hi} Qa-2$^-$
HSA$^+$, CD8^{hi} CD4^{lo} CD3-TCR^{hi} Qa-2$^-$
HSA$^{+/-}$, CD8$^-$, CD4^{hi} CD3-TCR^{hi} Qa-2$^+$
HSA$^{+/-}$, CD8^{hi} CD4$^-$ CD3-TCR^{hi} Qa-2$^+$
CD8$^-$ CD4$^+$
CD8$^+$ CD4$^-$
Mature peripheral CD3-TCR^{hi} cells

Fig. I.1. A diagrammatic summary of the phenotypic stages of the intrathymic developmental pathway for T cells in mice. BM: bone marrow. X: positive selection. ?: possible positive selection. DN: double negative (Adopted from J. Nikolic-Zugic, 1991. *Immunol. Today*, 12:65-70).

intermediate immature CD3$^-$ stages bearing only low levels of either CD8 or CD4 (Wu et al, 1991). Induction of the TCR gene rearrangement and the expression of TCR on the cell surface in association with CD3 is a major event in the thymus, which happens at the early double positive stage. About the time TCR is expressed, CD4 and CD8 are also expressed at detectable levels. These cells are still proliferative (Nikolic-Zugic and Bevan, 1988) for a few divisions, and might constitute the majority of dividing cells in the thymus (Scollay, 1991). About 80-85% of thymocytes are double positive cells, which accounts for the majority of the cortical thymocytes. However, the proliferative capacity of those cells decreases as the level of IL-2R expression falls and CD4, CD8 and TCR levels increase, possibly through some unidentified intermediate stages.

Histologically, the thymus is divided into the peripheral cortex and the central medulla. In young animals the medulla comprises about 15% of the thymus tissue. Various stages of the developing thymocytes localize at different thymic compartments, which contain different thymic epithelia (van Ewijk, 1991). Thymic epithelial cells are organized into a network, with thymocytes interspersed between them. The epithelial cells and the bone marrow derived dendritic cells express class I and class II molecules. Interestingly, some thymic dendritic cells also express IL-2 receptor and CD8 (Ardavin and Shortman, 1992). Soon after the appearance of the TCR on the cell surface, a selection event occurs (Jenkinson et al., 1990). Cells cease to mature if they bear receptors that recognize and strongly react to self antigens, a process is referred to as *negative selection*. Cells with weak reactivity to self antigen and self MHC are selected to generate a peripheral functional T cell repertoire, which is ready to respond to invading antigen in association with self MHC. This maturation and acquisition of the MHC restriction process is termed *positive selection*. Both types of selection take place through the interaction of self peptides bound to MHC class I and class II molecules on the surface of thymic epithelial cells and the TCR, CD4 and CD8 molecules on the thymocytes. These interactions are assisted by cell adhesion molecules, such as CD2, LFA3, ICAM-1, Mac-1 or CD44. The affinity of the interaction probably determines whether positive, negative or no selection occurs (Golub and Green, 1991).

C. Positive Selection

T cells preferentially recognize peptides associated with self MHC. Our understanding is that this bias in MHC restriction is learned during T cell development in the thymus. Immature T cells move along the T cell developmental pathway and mature if they are able to recognize but not react strongly to self-MHC. This has been termed positive selection of self-MHC reactive T cells. At the same time, if these cells respond too weakly (perhaps due to lack of stimulation), or too strongly to self MHC (with or without self antigens), they will die (Rothenberg, 1990). Thus, positive selection is a key step in the development of a self-MHC restricted mature T cell repertoire for the immune system.

D. Negative Selection

The observations that thymocytes are rapidly proliferating and only a small number of them enter the periphery suggest that a large number of them are lost and a selection process occurs in the thymus (Jerne, 1971). It is known that some of this loss happens during T cell receptor rearrangement; those cells without successful rearrangement will probably die. One example of this is that thymocytes in severe combined immunodeficiency (SCID) mice, which are defective in the V(D)J recombinase and thus lack the ability to rearrange the T cell receptor, are aborted at the early stages (Ansell and Bancroft, 1989). Cells with successful rearrangement of T cell receptor genes are then submitted to the subsequent selection process. Jenkinson et al. (1990) showed that as soon as T cell receptor is expressed, thymocytes are capable of being negatively selected. This selection process has been suggested as important in maintaining self tolerance (Hengartner et al., 1988).

Theoretically, one mechanism of T cell tolerance could be that a T cell (or a clone of T cells) that bears an antigen receptor that reacts with self antigens plus self MHC is lost or deleted during development. The concept of clonal deletion of self-reactive lymphocytes was postulated by Burnet in the 1950s, but only recently, with the development of advanced techniques, has it been possible to demonstrate this. One such advance has been the breeding of transgenic mice that have been genetically engineered to express certain defined TCR genes. Another advance has been the production of monoclonal antibodies to different portions of the variable region of T cell receptor.

Evidence for the existence of a negative selection process was first demonstrated using a type of antigens called superantigens, which specifically stimulate majority of T cells bearing a particular T cell receptor Vβ region. These responding cells represent a significant percentage of the total T cells in an individual, while a conventional antigen only stimulates a very small percentage of total T cells. There are two general groups of superantigens, those expressed endogenously in an individual, for example, MMTV encoded superantigens, and those derived exogenously, such as staphylococcal enterotoxins. Anti-TCR Vβ region antibodies were first used to demonstrate clonal deletion as a mechanism of T cell tolerance with Vβ17$^+$ cells, which frequently recognize I-E plus a B cell derived product (believed to be an MMTV product). It was found that Vβ17 positive cells were absent in the peripheral T cell repertoire and in the medullary T cells in I-E expressing mice, although the percentage in the immature T cell population bearing Vβ17 was not altered (Kappler et al., 1987). Therefore, clonal deletion of autoreactive T cells by superantigens probably occurred during the transition from immature double positive T cells to relatively mature single positive T cells in the thymus. The deletion pattern was dominantly inherited, which accords with the inheritance pattern of MHC.

Clonal deletion was subsequently shown with the Vβ dominant recognition of another set of endogenous superantigens called Mls [minor lymphocyte stimulatory] which are products of MMTV (Janeway, 1991) presented by an MHC class II molecule. Different types of MMTV can be found in different mouse strains and each of them encodes a different Mls phenotype, which in turn is recognized by T cells using different Vβ genes. For example, Mls-1^{a} is predominantly recognized by those cells expressing either Vβ8.1 or Vβ6. It has been shown that in Mls-1^{a} mice such potentially self-reactive T cells normally exist in the immature thymic T cell population, but fail to mature into peripheral T cells (Blackman et al., 1990). The injection of exogenous superantigen, staphyloccocal enterotoxin B, or the addition of the toxins to fetal thymus organ culture also resulted in the elimination of T cells bearing a particular set of Vβ receptors from the mature T cell pool (White et al., 1988; Finkel et al., 1989; Jenkinson et al., 1989).

Overall, with all the superantigens defined so far, clonal deletion of potentially reactive T cells occurred at immature stages during development in the thymus. Many researchers believe that the same applies to any antigen in the body. However, recent experiments have demonstrated that superantigens do not interact with MHC in the same way as conventional antigens, and thus this behavior may not be assumed to represent the general situation (von Boehmer, 1991).

In T cell receptor transgenic mice, most of the T cells express the same T cell receptor. One such mouse was created by using T cell receptor α and β chain genes from a CD8^{+} cytotoxic T cell clone with specificity for H-2D^{b} plus an endogenous antigen called H-Y (male antigen) (Kisielow et al., 1988). This antigen is expressed in male, but not female mice. In the resulting female H-2D^{b} mice, CD8 single positive T cells bearing the transgenic receptor developed normally. However, in male H-2D^{b} animals, where H-Y is a self antigen, few thymocytes at the double positive stage could be found and few matured beyond this stage. This clearly demonstrated that thymocytes which display T cell receptors with anti-self activity are lost at some immature stage(s) during maturation. This phenomenon has also been demonstrated with the TCR transgenes of a T cell clone derived from BALB.B anti-BALB/c mixed lymphocyte reaction specific for L^d. When transgenic mice express L^d, functional mature T cells bearing the transgenes were deleted (Sha et al., 1988). OVA specific T cell receptor genes have also been utilized as transgenes and have confirmed the deletion of autoreactive T cells during development in the thymus (Murphy et al., 1991).

Accumulating evidence suggests that clonal deletion occurs at the double positive stage. It is known that an MMTV product plus I-E molecules can be recognized by $V\beta17^+$ $CD4^+$ T cells, but not $V\beta17^+$ $CD8^+$ cells (Burgert, 1989). However, in I-E^+ mice deletion of $V\beta17^+$ cells occurred in both $CD4^+$ cells and $CD8^+$ cells. The simplest explanation for this observation is that the $CD8^+$ $V\beta17^+$ T cells are deleted when they express CD4, that is, at the double positive stage. This was confirmed by injecting animals with anti-CD4 antibody, which interferes with the recognition of class II MHC by $CD4^+$ thymocytes. After anti-CD4 treatment, $CD8^+$ $V\beta17^+$ cells were able to mature in I-E positive mice, demonstrating that negative selection event is probably occurred at a stage when the cells bear both CD4 and CD8. Similarly, Hengartner et al. (1988) reported that clonal deletion of $V\beta6$ cells in both $CD4^+$ and $CD8^+$ cells can be prevented by *in vivo* administration of anti-CD4.

Cells derived from bone marrow are sufficient to induce clonal deletion. Experiments with chimeric mice using bone marrow of an I-E^+ donor in a host lacking I-E still demonstrated the deletion of $V\beta17^+$ cells, suggesting that the signal (I-E) for the negative selection of $V\beta17^+$ T cells can be provided by the thymic cells derived from bone marrow. Recent experiments have also proven that thymic cortical epithelial cells are effective in processing and presenting self antigens to T cell hybridomas (Lorenz and Allen, 1989). This indicates that thymic epithelial cells may also be capable of inducing negative selection. One experiment with deoxyguanosine treated thymus directly demonstrated that thymic epithelial cells were effective in eliminating Mls reactive cells (Webb and Sprent, 1990). Thus both thymic epithelial cells and myeloid thymic resident cells are probably able to initiate negative selection.

To find out where the negative selection event actually occurs, Hengartner et al. (1988) used immunostaining of thymus cryosections and cytofluorometric analysis to show that, in animals which do not express Mls-1^{a}, Vβ6 cells were not deleted and staining could be seen on cells in both the cortex and medulla of the thymus. On the other hand, in animals expressing Mls-1^{a}, Vβ6 cells could only be seen in the cortex, not the medulla. Thus, negative selection must occur at the boundary between the thymic cortex and the medulla. As the medulla is known to be populated mainly with mature T cells, it seems that negative selection occurs prior to maturation. However, Murphy et al. (1990) have shown that, in OVA specific T cell receptor transgenic mice, the *in vivo* administration of OVA peptide induced cell death throughout the cortex of the thymus.

Turka et al. (1991) recently reported that cross linking of CD3 with CD4 or CD8 synergized signal transduction and increased expression of CD28 in CD4^{+}CD8^{+}CD28^{-}/dull human thymocytes. Subsequent incubation with soluble anti-CD28 led to proliferation. CD28 has no effect on cells stimulated with anti-CD3 alone. It is further demonstrated that BB-1, the natural interaction counterpart for CD28, is expressed on thymic stromal cells. This led the authors to suggest that CD28 ligation leads to positive selection, whereas in the absence of CD28 stimulation cells go through negative selection. Nieto et al. (1990) have suggested that in human thymocytes the lack of IL-2 production after activation may trigger those cells to be negatively selected. Research into the role played by regulatory molecules will provide more information for us to understand T cell development.

E. Cell Death

Cells, like living organisms of which they are a part, go through a cycle of growth, reproduction and death. According to morphological and biochemical changes that take place in the process, the death of nucleated eukaryotic cells can be classified into two major types: necrosis and apoptosis (Wyllie et al., 1980). Necrosis can be induced by injuries such as complement attack, hypotonic and hypertonic shock, heat shock, hypoxia, toxin exposure and lytic virus infection. The necrotic process begins with cytoplasmic membrane damage, leading to changes in the permeability of the membrane to cations and in the distribution of water. Characteristic morphological changes associated with necrosis include swelling and rupture of plasma and organelle membranes, loss of cytoplasmic organization, dilation of endoplasmic reticulum, swelling of mitochondria, and relatively maintianed nucleus structure. The released cell contents, including lysosomal enzymes, can further damage neighboring cells, leading to the death of cells in groups. However, this thesis is designed to study physiological cell death in immature T cells, therefore, the necrotic type of cell death will not be discussed further.

Apoptosis (pronounced with the second "p" silent) was coined by Kerr et al. (1972) from the Greek word αποπτωσισ, meaning the falling of petals from a flower or leaves from a tree. It describes physiologically programmed cell death, a self-destructive, active suicide process. The characteristic changes associated with apoptosis are condensation of chromatin and loss of nuclear structure, and the formation of cytoplasmic membrane blebs and apoptotic bodies which is a portion of cytoplasma surrounded by intact cell membrane and may contain cell organelles or fragments of nuclei. Apoptosis also involves fragmentation of genomic DNA into an oligonucleosomal ladder (which is not observed in the necrotic process). In contrast to necrosis, changes in cytoplasmic organization cannot be observed in the early stages of apoptosis. In many cases, apoptosis depends upon RNA and protein synthesis by the dying cell, and is thus considered a suicide process. Another difference between apoptosis and necrosis is that the latter often affects groups of cells and induces accompanying inflammatory responses while apoptosis does not. In tissue, apoptotic cells and apoptotic bodies are rapidly phagocytosed by local phagocytic cells such as macrophages. This might explain why only a few or no apoptotic cells and apoptotic bodies are observed at any given time in tissues undergoing apoptosis. Phagocytosis is believed to be mediated by newly exposed polysaccharide groups on apoptotic cell surface (Duvall et al., 1985) and the adhesion molecules on macrophages (such as vitronectin receptor, Savill et al., 1990).

Apoptosis occurs in embryogenesis, metamorphosis, hormone-induced tissue atrophy, and tissue turnover. It is also observed during pathological changes such as tumor regression, malnutrition and some viral infections (such as in HIV infected human T lymphoblasts). During mammalian development, apoptosis has been described as a mechanism for tissue modeling or molding, such as in deletion of redundant epithelium after fusion of the palatine processes (Hindrichsen and Stevens, 1974), cell deletion during spontaneous metamorphosis of tadpole tail (Kerr et al., 1974), formation of intestinal villi (Harmon et al., 1984), and differentiation of the retina (Young, 1984). During spermatogenesis and functioning of the immune system, apoptosis, as a counterbalance to mitosis, has particular significance in maintaining cell homeostasis. Apoptosis is also associated with disease states, such as nerve degeneration in staggerer mice (Sonmez and Herrup, 1984).

The study of the mechanism of apoptosis has been hampered by a lack of suitable systems. In most cases, apoptosing cells are scattered among viable cells, and even when apoptosis has been enhanced by a defined stimulus the onset of apoptosis is not synchronized among the cells. Moreover, biochemical processes taking place in apoptosis cannot be studied with intact tissue, because apoptotic cells are rapidly taken up by phagocytes in tissue. Thus, most of the limited data on the mechanism of apoptosis has been generated with *in vitro* tissue culture involving such phenomenon as thymocyte death induced by glucocorticosteroids. However, cells undergoing apoptosis *in vitro* are not removed by phagocytic cells; instead they ultimately swell and lyse, a terminal phase of apoptosis *in vitro* that has been referred to as secondary necrosis (Cotter et al., 1990). Thus this limitation should be borne in mind when one interprets the data obtained *in vitro*.

It has generally been accepted that apoptosis requires protein synthesis following the observation that DNA fragmentation and cell death can be inhibited by cycloheximide and actinomycin D, as in glucocorticoid (Wyllie et al., 1984), radiation (Sellins and Cohen 1987), activation (Ucker et al., 1989) and calcium ionophore (McConkey et al., 1989; Kizabi et al., 1989) induced cell death in thymocytes. However, recently it has been demonstrated that inhibition of protein and RNA synthesis can also induce apoptosis (Martin et al., 1990). This seems to be conflicting with observations that apoptosis requires protein and RNA synthesis, but it may be an effect when death is evaluated after treatment with the inhibitors. It is probable that in these cells the proteins that cause cell death normally exist, but its function is checked by a short lived survival signal. Blockage of RNA or protein synthesis eliminates the survival signal and cause cell death. Cohen et al. (1992) suggested that the "death gene" product in these cells is already expressed, but the activity is held in check by shorted lived inhibitory proteins. The rapid decay of the inhibitory proteins leads to cell death after inhibition of protein synthesis. Target cell death induced by CTL and NK cells is rapid and does not involve *de novo* protein synthesis in target cells (Duke et al., 1983; L. Shi et al., 1992).

One hallmark of apoptosis, in contrast to necrosis, is the fragmentation of genomic DNA into single and multiple nucleosome-sized fragments. It is sensible to predict the involvment of a nuclease in this process. Indeed, it has been reported that microccocal nuclease digestion of normal thymocyte nuclei demonstrated characteristic chromatin condensation and nucleosomal-sized DNA cleavage (Arends et al., 1990). There have been many attempts to isolate the nucleases involved in apoptosis. Giannakis et al. (1991) demonstrated that the Ca^{++}/Mg^{++} dependent nuclease activity increased about 6-fold during colchicine induced apoptosis in chronic lymphocytic leukemia cells. Wyllie et al. (1986) showed that the extractable Ca^{++}/Mg^{++} dependent endonuclease activity rose from low levels, peaking as the morphology changed in lymphoid cell lines upon glucocorticoid treatment. Similarly, Kyprianou et al. (1988) found that there was 2-fold increase in Ca^{++}/Mg^{++} dependent nuclease activity in rat prostate within the first day following castration. One description of endonuclease expression in thymocytes following induction with glucocorticoid (Compton and Cidlowski, 1987) has been shown to be artifactual and due to the false identification of nuclease activity with the participation of histone released following DNA degradation (Alnemri and Litwack, 1989; Baxter et al., 1989). However, Gaino and Cidlowski (1991) have again confirmed that the previously purified nuclease from glucocorticoid activated rat thymocytes is distinct from histone and showed characteristic nuclease activity. Cohen and Duke (1984) have shown that an endonuclease with the capacity of generating DNA ladder is present in isolated nuclei from uninduced thymocytes, splenocytes and lymph node cells, but not in those from bone marrow leukocytes or Con A induced blasts. They and Orrenius et al. (1989) suggest that nuclei contain a Ca^{++}, Mg^{++}-dependent endonuclease, inhibited by Zn^{++}, that is responsible for DNA fragmentation in apoptosis. Interestingly, Crompton (1991) demonstrated that apoptotic cell death of IL-3 dependent cell line upon IL-3 deprivation could be inhibited by nuclease inhibitor aurintricarboxylic acid, suggesting a role of DNA fragmentation in apoptosis.

Apoptosis in many cell types involves the binding of the insulting reagent to the cell surface. Some of the secondary signals occurring between cell surface and the activation of nuclease have been identified. Pertussis toxin, a known inhibitor for GTP binding protein, could inhibit TNF mediated cytotoxicity (Hepburn et al., 1987). The PKC inhibitor H-7 has been shown to inhibit glucocorticoid induced thymocyte death (Ojeda et al., 1990). How these secondary signals relate to apoptosis needs to be further defined. Many workers assume that cell death by apoptosis follows a common pathway independent of the nature of the stimulus and the cell types involved. Hence, it is possible that identification of the biochemical events associated with apoptosis could provide information for biological research and the clinical treatment of a number of diseases.

It has been shown in some instances that apoptosis require calcium. It was first appreciated that calcium influx in hepatocytes followed by treatment with toxin is associated with cell death. Bachvarof et al. (1977) observed that the incubation of isolated mouse spleen nuclei with Ca^{++} and Mg^{++} resulted in DNA fragmentation characteristic of that observed during apoptosis, which has been confirmed by Cohen and Duke (1984). Cell death in such systems has also been inhibited by the addition of Ca^{++} chelators, such as EGTA or EDTA. A rise in intracellular calcium has been shown to be associated with glucocorticoid induced killing in thymocytes (Cohen and Duke, 1984) and activation-induced cell death in immature thymocytes (Finkel et al., 1987). However, an elevated Ca^{++} level is not enough to cause DNA fragmentation until significant membrane damage has occurred (Nagelkerke et al., 1989). Although killing by cytotoxic T cells and by tumor necrosis factor shows typical DNA fragmentation and nuclear condensation, elevation in Ca^{++} concentration is not observed (Haseqawa and Bonavida, 1989). In addition, elevation of Ca++ has been found to be associated with necrotic cell death (Waring et al., 1991). Hence, the role of calcium in apoptosis remains unclear.

F. Apoptosis and T Cells

In vitro culture of thymocytes is a widely used model system for studying apoptosis. Thymocytes have been induced to undergo apoptosis by treatments with glucocorticoids (Wyllie, 1980), low-dose γ-irradiation (Sellins and Cohen, 1987), dioxin (McConky et al., 1988), or the calcium ionophore A23187 (McConky et al., 1989c). It has been demonstrated that apoptosis can be induced by stimulating thymocytes in fetal organ culture, using either antibodies to CD3 (Smith et al., 1989) or the superantigen, staphylococcal enterotoxin B (Jenkinson et al., 1990). Similarly, when thymocytes from H-Y specific TCR transgenic female mice were cultured in suspension with APCs from syngeneic male mice, there was a significant antigen specific induction of apoptosis in $CD4^+CD8^+$ cells (Swat et al., 1991). MacDonald and Lees (1990) showed that $CD4^+CD8^-V\beta6^+$ neonatal thymocytes were specifically deleted in *in vitro* culture of $Mls\text{-}1^a$ mice. Thus it is clear that autoreactive T cells are deleted at immature stages in the thymus.

Another phenomenon involving apoptosis and T cells occurs when cytotoxic T cells kill their target cells, inducing DNA fragmentation and morphological changes in the target cells that resemble apoptosis (Duke et al., 1983). Characteristically, target killing by cytotoxic T cells does not require protein synthesis in the target cell (Duke et al., 1983). It has been suggested that the killing factor is produced by cytotoxic T cells and transferred to the target (Henkart, 1985; Podack et al., 1991). Recent studies have suggested that substances in cytotoxic granules (Hayes et al., 1989; L. Shi et al., 1992) induce DNA fragmentation.

Newell et al. (1990) found that pretreatment with antibodies to CD4 made mature T cells die upon ligation of T cell antigen receptor with antibody to the CD3 complex. The authors suggest that CD4 is critical in determining the outcome of signals generated through TCR and might explain why the induction of effector T cells needs to be MHC restricted. Kawabe and Ochi (1990) reported that *in vivo* administration of staphylococcal enterotoxin B induced apoptosis in mature lymph node T cells, and suggest that this phenomenon may represent a mechanism of peripheral T cell tolerance. However, in this experiment the investigators observed cell death on the 4th day, rather than 12-24 h after staphylococcal enterotoxin B injection. They could not observe cell death in mature T cells within a few hours post activation, as they did in the immature T cell population in the thymus (Ochi, personal communication). A possible explanation for the death of lymph node cells is lack of stimulus and lymphokine support for these stimulated blasts 4 days after the initial stimulation (Cohen et al., 1991). Therefore, the phenomenon of activation-induced cell death in mature T cells under physiological conditions needs to be further confirmed.

Negative selection, we have seen, seems to involve the presentation of self antigens by bone marrow-derived thymic dendritic cells to a double positive thymocyte. If the thymocyte responds to the presented antigen, it dies, whereas mature T cells, upon recognition of presented antigen, become activated, release lymphokines, express lymphokine receptors, and proliferate. What is special about antigen presentation in the thymus which results in the death of the cells? One possible explanation is that the antigen presenting cells (the thymic dendritic cells) have the ability to kill any thymocyte that recognizes the MHC (plus processed antigen) on the dendritic cell surface. This would be analogous to the action of cells called veto cells which appear to have just this type of activity (Fink, et al., 1988). However, it has now been shown that thymic dendritic cells are good antigen presenting cells for mature T cells, appearing to lack any special killing or inhibitory activity. Among different thymic antigen presenting cells tested for their ability to induce negative selection in thymocytes developing in thymic organ cultures, and for their ability to stimulate mature T cells, dendritic cells appeared to be good at both inducing negative selection and stimulating mature T cells (Matzinger and Guerder, 1989; Lorenz and Allen, 1989.). Thus, it does not seem that a specialized antigen presenting cell exists that can specifically destroy the responding immature T cells. An alternative view is that it is not the antigen presenting cell but the immature thymocyte that responds to presented antigen specifically. It has been shown that activation of developing thymocytes in organ culture causes them to undergo apoptosis (Smith et al., 1989). Thus, it seems as though activation of a double positive thymocyte causes the cell to commit suicide.

G. Thesis Objectives and Rationales

This thesis is designed to study the mechanism of negative selection. As previously discussed, a large number of cells disappear during T cell development in the thymus, and this loss has been attributed to negative selection. Negative selection has been proposed as a mechanism of somatic learning of immune tolerance (Nossal, 1983). However, little is known about how it occurs. Our working hypothesis is that, during T cell development, these autoreactive T cell clones, which have been generated by random T cell antigen receptor gene rearrangement, enter a suicide pathway after being activated by self antigens at immature stages during the development. This is in contrast to mature T cells which have become resistant to activation-induced cell death. This hypothesis was tested by the following approaches:

1. Activation-induced cell death in the thymus is difficult to study under natural conditions because the number of cells with specificity to a given self antigen is limited. It was postulated that if a polyclonal activator could be used, then many clones could be activated to enter the suicide pathway. As it is known that monoclonal antibody to CD3 can nonspecifically activate mature T cells and hybridomas (Leo et al., 1987), this antibody was selected as the polyclonal activator for our study. We found that after *in vivo* injection of anti-CD3, many cells in the thymus were induced

to undergo apoptosis as observed by DNA fragmentation, and cellular structural changes (Shi et al., 1991).

2. Due to the instability of thymocytes *in vitro*, especially those bearing both CD4 and CD8 markers, it is difficult to further analyze activation-induced cell death with thymocytes. We postulated that an *in vitro* model would help us immensely in our study of activation-induced cell death. It has been shown that some T cell hybridomas cease to proliferate after activation (Ashwell et al., 1987). It seemed possible that the lack of proliferation of the hybridoma cells was due to activation-induced cell death and thus could be used as an *in vitro* model. A1.1 is a T cell hybridoma produced by fusion of a poly18-specific $CD4^+$ T cell and thymoma BW5147 (Fotedar et al., 1985). We activated A1.1 cells with antigen presented by a B cell lymphoma (TA3), with anti-CD3 monoclonal antibody or with other mitogenic stimulators, and demonstrated that activation of A1.1 cells induced apoptosis, and at the same time released growth factors. The activation-induced cell death of the T cell hybridoma was dependent on macromolecular synthesis (Shi et al., 1990).

3. It is known that CsA exerts its effect in the immune system by inhibiting activation of T cells (Halloran and Madrenas, 1991). We found that CsA completely inhibited activation-induced cell death both in thymocytes *in vivo* and in T cell hybridomas *in vitro* (Shi et al., 1989), a finding which could shed light on why some patients develop autoimmunity following administration of cyclosporin A. In addition, these experiments provided evidence for a link between activation-induced cell death and negative selection.

4. Activation-induced cell death through the T cell receptor is a complicated process which requires many signal transduction steps from the process of binding to the cell surface receptor to the activation of the endogenous nuclease. Characterization of the pathway will undoubtedly provide information on the mechanism of apoptosis. By using antagonist and agonist, we showed that both

PKC activation and Ca^{++} are required for activation-induced cell death.

5. Genomic DNA fragmentation is generally recognized as a characteristic of apoptosis. However, there is no information on the relationship between apoptosis and DNA fragmentation. For example, does DNA fragmentation lead to cell death, or vice versa? To address this question, a known nuclease inhibitor, aurintricarboxylic acid (ATA), was included in our studies. This substance completely inhibited cell death *in vitro* and *in vivo*. Kinetic studies show that ATA exerts its effect at or near the time of DNA fragmentation. These and our observation about the ability of ATA to inhibit DNA fragmentation in isolated nuclei exposed to Ca^{++} and Mg^{++}, strongly suggest that ATA prevents apoptosis by inhibiting endogenous endonuclease activity, and thus indicate that DNA fragmentation leads to cell death.

6. Activation could occur in many possible ways. Many protooncogenes have been identified to be associated with cell activation and proliferation, but little is known about the function of these protooncogenes. It was thought that some of the nucleus-associated protooncogene products may be involved in the activation of the nucleases. Antisense oligonucleotides corresponding to protooncogene messengers provided a method to investigating this. Of several antisense oligodeoxynucleotides tested, antisense to *c-myc* was found to prevent cell death (Shi et al., 1992), providing an important clue toward understanding mechanism of apoptosis.

II. MATERIALS AND METHODS

A. Animals

Male BALB/c mice were obtained from Ellerslie Animal Farm, University of Alberta, Edmonton, Canada. They were maintained at the University of Alberta Health Science Animal Services and were allowed to rest for 1 week following shipment. Mice were age matched (4-6 weeks old) in each experiment. Food and water were provided *ad libitum*.

B. Reagents

Chemicals

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], FITC (fluorescein isothiocyanate conjugate), cycloheximide, actinomycin D, ConA (concanavalin A), PMA (4-β-phorbol 12 myristate, 13-acetate), calcium ionophore A23187, and ionomycin were purchased from Sigma (St. Louis, MO). ATA (aurintricarboxylic acid) was obtained from Aldrich (Milwaukee, WI). Cyclosporin A was provided by Dr. Paetkau's laboratory and Sandoz Canada Inc. (Dorval, Quebec). Staurosporin was obtained from Kamiya (California). H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) was obtained from Seikagaku America Inc. (St. Petersburg, FL). All other chemicals were the purest grade available and were obtained commercially.

Antigen

The nonrandom synthetic *c-myc* peptide, poly 18 {poly [Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$]}, and poly 18 derivatives were synthesized according to Barton et al. (1977) and kindly provided by Dr. B. Singh (University of Alberta). They were dissolved in saline and pH was adjusted to 7.2.

Oligodeoxynucleotides

Synthetic antisense oligonucleotides were complementary to oncogene sequences extending from the ATG starting codon to 15 or 21 nucleotides downstream (Table IX.1). All oligodeoxynucleotides were synthesized by the Regional DNA Synthesis Facility at the University of Calgary, Calgary, Canada. In order to provide large quantities, the oligonucleotides were not gel-purified in most of the cases. Contaminating n-1 and n-2 sequences were present at a concentration of approximately 10% (quality control information). To improve the biological efficiency, the oligodeoxynucleotides were substituted with a sulphur-for-oxygen at phosphorus position. The phosporothioate oligodeoxynucleotide analogues were used in the same way as conventional oligodeoxynucleotide, except at lower concentrations. All of the oligonucleotides were dissolved in double-distilled water as 1 mM stock solution.

Cytokines

Tumor necrosis factor α (TNFα) was obtained from Dr. Guilbert's laboratory, Canadian Red Cross, Edmonton, Canada. Transforming growth factor β1 (TGF-β1) and transforming growth factor β2 (TGF-β2) were obtained from the R & D system.

C. Cell Lines

T cell hybridoma A1.1 and B1.1 were originally obtained from Dr. B. Singh's laboratory, University of Alberta (Fotedar et al., 1985). They were recloned and selected for responsiveness to activation stimulus and T cell receptor expression. Parental thymoma BW5147 and antigen presenting cell TA3 (Glimcher et al., 1983) were also obtained from Dr. B. Singh. A hamster anti-murine CD3 B cell hybridoma,145-2c11 (Leo et al., 1987) and a hamster anti-murine T cell receptor β chain B cell hybridoma, H57-597 (Kubo et al., 1989) were used as sources of antibodies to the T cell receptor complex. All cells were cultured at 37 ^{o}C in humidified atmosphere containing 5% CO_2 in RPMI-1640 (GIBCO Laboratories, Grand Land, NY) supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 uM 2-mercaptoethanol, 5-10% 56 °C-inactivated fetal bovine serum and penicillin-streptomycin.

D. Radiolabeling of Antibodies

Protein A purified anti-CD3 or anti-TCR was labelled with ^{125}Iodine using iodobeads (Pierce Chemical Co., Rockford, IL), as follows. Purified antibodies (100 µg) were labeled with 0.5 mCi ^{125}Iodine (New England Nuclear, Boston, MA) for 100 minutes in the presence of one iodobead. The labelled antibodies were then separated using Sepharose G-25. For tracing antibody *in vivo,* labelled antibody 1×10^6 to 5×10^6 counts per minute (cpm) was mixed with 100 µg of unlabeled antibody and injected i.v. or i.p.

E. Cell Activation Protocols

1. *In Vivo* Thymocyte Activation

Four to five week-old male BALB/c mice were intraperitoneally injected with 200-300 µl $(NH_4)_2SO_4$-precipitated normal hamster serum (NHS), rat anti-mouse L3T4 (CD4) (from B cell hybridoma GK1.5), or hamster anti-mouse CD3 (from B cell hybridoma 145-2c11). Depending on the type of assay, at different times post treatment ranging from 10 to 40 h, spleen, mesenteric lymph nodes and thymus were removed and assayed for the biological effects of activation reagents.

2. *In Vitro* Activation of T Cell Hybridomas

T cell hybridomas were activated with specific antigens as previously described (Fotedar et al., 1985). Briefly, 1×10^5 hybridoma cells were cocultured with 2×10^4 antigen presenting cells (TA3) in the presence of 5 µg of poly-18 or poly EYA in 200 µl media. Alternatively, T cell hybridoma cells were activated by different concentrations of ConA or a combination of PMA and ionomycin. T cell hybridomas were also activated by anti-CD3 coated tissue culture plastic. Tissue culture plastic was coated with anti-CD3 by incubating the plastic with 0.05 M Tris-HCl (pH

9.0) overnight at 4 °C or for 1 h at 37 °C. Unbound antibody was washed away with PBS. In all cases, cells were cultured in RPMI 1640 supplemented with 5% 56 °C-inactivated FCS and incubated at 37 °C, in humidified 5% CO_2/95% air, for the times required.

F. Fluorescent Marker Analysis

Thymocytes from each thymus were counted, then stained with FITC-rat anti-mouse CD8 and PE-rat anti-mouse CD4 (Becton Dickinson, Mountain View, CA). Alternatively, cells were incubated with anti-CD3 (145-2C11), followed by FITC-rabbit anti-hamster antibody. All of the staining steps were at 4 °C (on ice). Stained cells were fixed in 1% paraformaldehyde in PBS and analyzed using a FacScan analyzer (Becton Dickinson).

G. Cytokine Assays

The activation of T cell hybridoma A1.1 was demonstrated by the production of IL-2, which was measured by its ability to support an IL-2 dependent T cell clone, CTL.L. CTL.L cells were maintained in RPMI media containing PMA stimulated EL-4 supernatant as a source of IL-2. CTL.L cells were starved from IL-2 for 12 h before being used for assay. The assay was carried out in 96-well flat bottom plates. Cells (10^4 per well) were cultured for 24-48 h with certain concentrations of the primary A1.1 culture supernatant. The potency of the stimulation of CTL.L growth was measured by incorporating [^{3}H]-thymidine (40 kBq/well) for 6-12 h.

I. Cell Death Assays

1. Genomic DNA Isolation

Ten to 14 h after culture *in vitro* or treatment *in vivo*, approximately 5 x 10^7 A1.1 cells or thymocytes were harvested and washed twice with PBS at 4 °C and suspended in 50 µl PBS. Two ml of digestion solution (0.5 M EDTA, 0.5% sarcosyl and 200 µg proteinase K/ml) were added. Digestion was carried out at 50 °C for 3-4 h with occasional gentle swirling. Protein was removed from the digested solution by extracting with equal volume of equilibrated phenol, then equal volumes of phenol/chloroform and lastly with equal volumes of chloroform. The resultant DNA solution was dialysed against 6 L of STE (50 mM Tris.HCl, 10 mM EDTA, 10 mM NaCl, pH 8.0) at 4 °C. RNA was then removed by treatment with 200 μg RNase A/ml for 3 h at 37 °C. DNA was precipitated with 66% ethanol in the presence of 0.3 M sodium acetate at -20 °C overnight. The precipitate was collected and washed once with 80% ethanol. After drying, the precipitate was dissolved in TE (10 mM Tris.HCl, 1 mM EDTA, pH8.0) at the desired DNA concentration. Electrophoresis was carried out in TPE (80 mM Tris-phosphate, 2 mM EDTA, pH8.0). Twenty micrograms of DNA were loaded to each slot of an agarose gel. DNA bands were visualized by ethidium bromide staining.

2. Direct Isolation of Fragmented Genomic DNA

Single cell suspension was washed with PBS or BSS and then suspended in 0.3-0.5 ml of the washing buffer in a 15 ml polypropylene tube. Gentle vortexing was applied to ensure complete resuspension of cells. Three to 4.0 ml lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 1.0% Triton X-100 (avoid using ionic detergent)] was added while vortexing. The lysate was then incubated on ice for 20 minutes and centrifuged at 11,000 g, 4 °C for 20 minutes. The resulting supernatant was transferred to a new tube and digested with RNase A followed by proteinase K for 1 h each at 37 °C. Protein was extracted twice with phenol:chloroform, then once with chloroform alone. Following extraction, DNA was precipitated by adding 0.3M NaAc, pH 5.2, and at least two vol. of ethanol at -70 °C for 1 h, or -20 °C overnight. Precipitated DNA was centrifuged at 11,000 g 4 °C for 20 minutes, and then transferred into 1.5 ml Eppendorf tubes and washed twice with 80% ethanol. The pellet was dried on a 50 °C water bath for 10 to 20 minutes and dissolved in 20-50 μl TE buffer. The DNA was analyzed on agarose gels.

3. Quick Method for Assaying DNA Fragmentation in Total Genomic DNA

Cells (4-6 X 10^5) were harvested into an Ependorf tube and lysed with 30 µl of lysis buffer [20 mM EDTA, 100 mM Tris (pH 8.0), 0.8% (w/v) sodium lauryl sarcosinate, 5 mg proteinase K/ml]. The lysate was mixed by flicking the tip of the tube and then incubated in a 50 °C water bath for at least 1.5 h. After adding 0.2 mg/ml RNase A, the mixture was incubated in a 37 °C water bath for another 30 minutes. The resulting DNA solution was analyzed on agarose gels.

4. Quantitation of DNA Fragmentation with ^{125}IUDR Labeled Cells

Actively dividing cells were labeled at the concentration of 10^6 cells/ml with 1 µCi/ml of fresh ^{125}IUDR, at 37 °C for 8-10 h. Labeled cells were harvested and washed with cold media at least 3 times. Treatments were then carried out in 200 µl media in 96-well tissue culture plates. Six to 12 h post stimulation, genomic DNA fragmentation was assayed as follows. Cells were harvested in 1.5 ml Ependorf tubes and lysed by adding 900 µl lysis buffer (5 mM Tris (pH 7.4), 2 mM EDTA, 0.5% Triton X-100 (non-ionic detergent) (total volume of 1.1 ml). The Eppendorff tubes were then vortexed vigorously to ensure complete lysis of cells. After incubating on ice for 20 min, the tubes were centrifuged at 14,000 x g, for 20 min, in a microfuge. One milliliter of supernatant (contains fragmented DNA) was transferred to a new Eppendorff tube, leaving 100 µl supernatant with the pellet to ensure that the pellets were not co-transferred. The radioactivity of the supernatant and the pellet were measured with a gamma counter. The percentage of fragmentation was calculated by the following formula.

$$\% \text{ DNA fragmentation} =$$

$$100 \times [(\text{Supernatant count} \times 1.1)/(\text{Supernatant Count} + \text{Sediment Count})]$$

5. Quantitation of DNA Fragmentation with Ethidium Bromide

This method was adapted from Duke and Sellins (1989). Following activation, 1-1.5 x 10^6 cells were harvested into 1.5 ml Ependorf tubes. After washing with PBS, cells were suspended in 1.1 ml of lysis buffer [5 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.5% Triton X-100]. After vigorous vortexing, the tubes were incubated on ice for 20 min. Following centrifugation at 14,000 x g for 30 min, 1 ml supernatant from each tube was transferred to a separate tube and 0.9 ml of lysis buffer were added to the pellets and sonicated to ensure solublization. Samples were then treated with 100 µg/ml of RNase A for 2 h at 37°C. 100 µl samples were diluted in 1.5 ml of ethidium bromide buffer (5 mM Tris-HCl, pH 8, 0.5 mM EDTA, 0.5 µg/ml ethidium bromide) and measured for DNA concentration with a fluorometer. The percentage of DNA fragmentation was calculated similarly as described above.

6. Cell Viability

MTT Staining

This method was essentially the same as Mosmann (1983). Briefly, 10^5 cells were treated in 200 µl media in 96 well plates. After required treatments, 100 µl supernatant from each culture were removed and 10 ml of MTT solution (5 mg MTT/ml in H_2O) were added and incubated at 37°C for 4 h and then100 µl of acid-isopropanol (0.04 N HCl in isopropanol) were added to each culture and mixed by pipetting or shaking on a plate shaker to dissolve the reduced MTT crystals. After all the crystals were dissolved, the relative cell viability was obtained by scanning with an ELISA reader with a 570 or 595 nm filter.

Trypan Blue and FITC Staining

Viability of individual cells was assayed by staining with 1:2 Trypan blue solution (GIBCO Laboratories, Grand Land, NY) and counted under a light microscope. Alternatively, the viability of individual cells was assayed by fluorescein exclusion. This was achieved by adding 10 µl of 500 µg/ml FITC to a 200 µl culture (containing 5% serum) and incubated at 37 °C for 15 minutes. After washing with PBS, the cells were fixed in 100 µl PBS containing 1% formalin and enumerated under a fluorescent microscope. Viability was calculated as percentage of nonfluorescent cells over total cell number.

J. Accessment of Nuclease Activity in Nuclei

1. Isolation of Nuclei

Twenty-five million A1.1 cells were harvested, washed once in PBS and pelleted. The pellet was then gently resuspended in 25 ml of ice cold lysis buffer [10 mM Tris-HCl, pH 7.4 with 2 mM $MgCl_2$, 1 mM dithiothreitol (added fresh) and 0.05 % Triton X-100]. The resulting mixture was then transferred into two 12.5 ml aliquots to an ice cold 15 ml Dounce homogenizer and gently passed 4 times with a type B pestle. The homogenate was then distributed to 15 ml conical centrifuge tubes by gently layering 5 ml aliqote over an equal volume of sucrose buffer [0.9 M sucrose + 10 mM Tris-HCl, pH 7.4 with 2 mM $MgCl_2$ and 1 mM dithiothreitol (added fresh)] and the tubes were centrifuged for 10 minutes at 1,500 rpm. The supernatants were subsequently aspirated off and the pellets were gently resuspended and combined in 6.25 ml of assay buffer [10 mM Tris-HCl, pH 7.4 with 2 mM $MgCl_2$] (i.e. approximately 4×10^7 nuclei/ml) and kept on ice.

2. Assay of Nucleus Endonuclease Activity

Aliquots (500 µl) of isolated nuclei (i.e. 2×10^7 nuclei) were combined in 1.5 ml Eppendorf tubes with different testing reagents in the assay buffer and brought to a final volume of 1.0 ml. The tubes were then allowed to incubate in a 37 °C water bath for 90 minutes.

3. Isolation of Fragmented DNA.

At the end of their incubation periods all tubes were centrifuged at 10,000 rpm for 15 minutes at 4 °C and the supernatants were carefully aspirated off. The pelleted nuclei were then gently resuspended in 400 µl of 10 mM Tris-HCl, 30 mM EDTA, 0.5 % Triton X-100, pH 7.8, by mixing with wide bore pipettes and then allowed to sit on ice for 20 minutes. The tubes were subsequently centrifuged at 10,000 rpm for 15 minutes at 4 °C. Supernatants (400 µl) were retreived without disturbing the pellet. Each supernatant tube was then treated with 20 µl of 10 mg /ml RNAse A (Sigma) and incubated for 3 h at 37 °C. Following this the supernatants received 40 µl of 0.01 M Proteinase K (E. Merck, Darmstadt) and were digested overnight at 50 °C. DNA was extracted by sequential treatments with equal volumes of phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform. DNA was precipitated overnight at - 20 °C. Resulting DNA samples were resuspended in 100 µl of TE buffer and analyzed on agarose gel.

K Histology Sections

Thymus tissue was fixed in 10% phosphate buffered formalin and embedded in paraffin. Six µm sections were stained with hematoxylin and eosin and viewed under a light microscope. Pictures were taken at x 900 magnification.

L. Ultrastructural Analysis

1. Transmission Electron Microscopy

Thymus fragments or A1.1 cells were fixed overnight at 4 °C in a freshly prepared mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.02% $CaCl_2$, pH 7.4 (Karnovsky, 1965). Tissue fragments or cell pellets were then washed 3 times with 0.1 M cacodylate buffer before postfixing with 1% OsO_4 in 0.1 M cacodylate buffer for 1 h at room temperature. This was followed by several rinses with 0.1 M cacodylate buffer, pH 7.4, then 0.1 M maleate buffer, pH 6.0. Next, 2% uranylacetate in 0.1 M maleate buffer, pH 5.2, was used as an en bloc stain, for one h at room temp (in the dark). Pellets were rinsed in 0.1 M maleate buffer, pH 6.0, then dehydrated by passing through concentrations of ethyl alcohol, and embedded in BEEM capsules in low viscosity resin (Spurr, 1969). Thin (60 nm) sections were double stained, first for 30 min with 2% uranyl acetate in 12.5% methanol and 35% ethanol (Kim et al., 1979), then for 10 min with Reynolds' lead citrate stain (Reynolds, 1963). Micrographs were obtained using an transmission electron microscope.

2. Scanning Electron Microscopy

Thymus pieces were harvested from mice 12 h post treatments with anti-CD3 or normal hamster serum. A1.1 cells were attached to a 0.01% poly-lysine coated microscope slide coverslip. The samples were fixed by 1% OsO_4 in cacodylate buffer for 30 min, then gradually dehydrated by being passed through increasing concentrations of ethanol, and stored in 100% ethanol. The samples were then dried by a critical point drying procedure, which involves substitution of ethanol with liquid CO_2 and raising the temperature until it is above the critical point at which instantaneous evaporation of CO_2 occurs. After being coated with gold, the samples were viewed with a scanning electron microscope.

M. Western Blot Analysis

Western immunoblotting was performed as described (Degols et al., 1991). A1.1 cells were cultured with antisense or nonsense *c-myc* phosphorothioate oligodeoxynucleotides for 18 hours. Cells were then washed with PBS and lysed with 10 mM Tris.HCl pH 8.3, 0.25 M Sucrose, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 0.5% NP40. Lysate supernatant was incubated with 50 µg/ml DNAse I at 4 °C for 30 minutes and protein was precipitated with 5 volumes of acetone at -20 °C. Pellets was dissolved in loading buffer and fractionated on 10% SDS polyacrylamide gel. After transfer to nitorcellulose paper, *c-myc* protein was detected with a rabbit antiserum against a conserved APSEDIWKKFELL *c-myc* peptide (provided by Dr. B. Singh, University of Alberta) and then ^{125}I-protein A. The staining was visualized by autoradiography.

N. Northern Blot Analysis

Total RNA were isolated as described (Chomczynski and Sacchi, 1987). Cells were pelleted and lysed in 4 M guanidine thiocyanate, 25 mM sodium citrate pH7.0, 0.5% sodium lauryl sarcosinate and 0.1 M 2-mercaptoethanol. The lysates were adjusted to a concentration of 0.2 M sodium acetate, extracted with phenol/chloroform/isoamyl alcohol mixture and were then precipitated with equal vol. of isopropanol at -70 °C. RNA samples were fractionated on 1% agarose/2.2 M formaldehyde denaturing gel, and transferred onto nylon membranes. The DNA probe used for *c-myc* detection was the XbaI and BamHI fragment from pSVc-myc-1 plasmid (Land et al., 1983). It was labelled with ^{32}P-dCTP by nick translation methods (BRL) according to manufacturer's instruction. Prehybridization and hybridyzation were carried out at 42 °C in a hybridization solution containing 5 X SSC (10 X SSC is 1.5 M NaCl, 0.15 M Sodium citrate), 2.5 mM EDTA, 0.1% SDS, 5X Denhardt's solution, 2 mM sodium pyrophosphate, 50 mM sodium phosphate and 50% formamide. After hybridization, the membranes were washed at high stringency with 0.2 X SSC, 0.1% SDS at 56 °C. The hybridized bands were visualized by exposed to X-ray films.

O. Statistics

With a few exceptions, most experiments in this thesis were repeated at least twice. Analysis of variance was performed using a Macintosh program, Statview 512^{+} (Brainpower, Inc.). Standard error is used to represent experimental variations throughout this thesis.

III. ACTIVATION-INDUCED CELL DEATH IN IMMATURE THYMOCYTES *IN VIVO*

A. *In Vivo* Antibody Tracing

As stated in the hypothesis, negative selection is a process by which autoreactive T cells are activated by self-antigens plus self-MHC, causing them to enter a suicide pathway at immature stages of development in the thymus. However, this hypothesis is difficult to test with conventional antigens *in vivo*, because the proportion of cells with specificity to a given conventional self-antigen among the total thymocytes is small. It is obviously desirable to have reagents with the capacity to activate a large number of cells in the thymus. Because antibodies to the T cell receptor complex, anti-CD3ε (Leo et al., 1987) and anti-TCRβ (Kubo et al., 1989) can polyclonally activate T cells and T cell hybridomas, we selected them as polyclonal activators to test our hypothesis. Therefore, the locations of these antibodies were traced to see whether they would reach the thymus after *in vivo* injection. Anti-CD3, anti-TCR and normal hamster IgG were purified by protein A affinity chromatography and then labelled with ^{125}Iodine. Mice were injected intraperitoneally (i.p.) with 6.3 x 10^6 cpm of radioiodinated anti-CD3, 5.4 x 10^6 cpm of radioiodinated anti-TCR, or 6.1 x 10^6 cpm of radioiodinated normal hamster IgG. At 6, 22, and 45 h post injection, thymus, liver, lung and mesenteric lymph nodes were removed from treated mice and the amount of ^{125}I-labeled antibody of each organ was assessed with a gamma counter. The data are shown in Table III.1 (3 mice/group at each time point). The amount of anti-CD3ε or anti-TCRβ antibodies that localize in the thymus varies from approximately 0.01% to 0.1% of the total injected and most of the labeled anti-CD3 and normal hamster IgG were cleared by 45 h post injection, while a significant amount of anti-TCRβ was still detectable, indicating different clearance rates for these antibodies. Both anti-CD3 and anti-TCR antibodies react with cells in the thymus (i.e., CD3$^+$ thymocytes), while normal Ig does not. As shown in Table III.1, more of the antibodies to TCR or

Table III.1. *In Vivo* Tracing of Antibodies to T Cell Antigen Receptor Complex

		Spleen	MLN[#]	Liver	Thymus	Lung
	Anti-CD3ε	8890 ± 1928	5862 ± 1063	61286 ± 14970	7036 ± 2201	19217 ± 4475
6 hrs	Anti-TCRβ	4829 ± 420	5262 ± 989	30848 ± 4053	2615 ± 208	5116 ± 431
	NHIg[§]	1184 ± 31	929 ± 227	7577 ± 653	676 ± 232	1805 ± 114
	Anti-CD3ε	4052 ± 917	2592 ± 801	29388 ± 5650	9255 ± 4442	11629 ± 3622
22 hrs	Anti-TCRβ	4598 ± 1374	3302 ± 1111	23184 ± 9246	1337 ± 521	3191 ± 1398
	NHIg	928 ± 96	523 ± 61	6778 ± 373	702 ± 143	999 ± 58
	Anti-CD3ε	495 ± 163	427 ± 172	5369 ± 1328	205 ± 100	1970 ± 1634
45 hrs	Anti-TCRβ	5044 ± 340	2965 ± 362	20028 ± 4254	1304 ± 407	5548 ± 2355
	NHIg	609 ± 109	498 ± 49	5767 ± 102	425 ± 93	960 ± 233

Groups of 3 Balb/c mice (4 week-old) were injected with ^{125}Iodinated protein A-purified anti-CD3ε (6.3 x 10^6 cpm), anti-TCRβ (5.4 x 10^6 cpm) or normal hamster immunoglobulins (6.1 x 10^6 cpm). Animals were terminated at different times post injection and the amount of radiolabeled antibodies in the different organs were counted with a gamma counter. Data presented are mean ± SE.

[#] mesenteric lymph node.

[§] normal hamster immunoglobulins.

CD3 were retained in the thymus than is normal hamster Ig, although similar amounts were injected. This shows that antibodies are able to cross the blood-thymus barrier to interact with thymocytes. To determine whether the radioactivity was in blood and tissue fluid or was bound to thymocytes, thymuses were dissociated into single cell suspensions and washed with nonradioactive media. There were significant amounts of labeled anti-CD3 and anti-TCR retained in thymocyte samples, but not normal hamster immunoglobulin (Fig. III.1). Further experiments demonstrated that no difference existed between i.p. injection and intravenous (i.v.) injection (Table III.2). Unless otherwise stated, all *in vivo* administration was through the i.p. route. Overall, these experiments demonstrate that antibodies to the T cell antigen receptor complex administered *in vivo* could reach thymocytes.

B. *In vivo* Administration of anti-CD3 Induces Cell Death in Thymocytes

Twelve hours post injection with anti-CD3 or normal hamster serum, mice were terminated and their thymuses removed and fixed for transmission electron microscopy. Thymuses from normal hamster serum-treated mice showed normal thymic morphology (Fig. III.2A). However, thymuses from anti-CD3 treated mice showed extensively condensed nuclei (Fig. 2B). Nuclear condensation is one characteristic of cells which have died by apoptosis (Wyllie et al., 1984). By scanning electron microscopy, control thymocytes (Fig. III.3A) were shown to have short microvilli characteristic of cortical thymocytes (Morris et al., 1984). Twelve hours after anti-CD3 stimulation, thymocytes lost the short microvilli and blunt, hemispherical to spherical protrusions of varying diameters appeared on the cell surface (Fig. III.3B). This change of surface morphology is often referred to as cell surface membrane blebbing, another characteristic of apoptosis (Wyllie et al., 1980). An additional characteristic of apoptosis is that genomic DNA is fragmented into a ladder of nucleosome-sized,180 to 200 base pair multiples (Wyllie et al, 1980). We therefore,

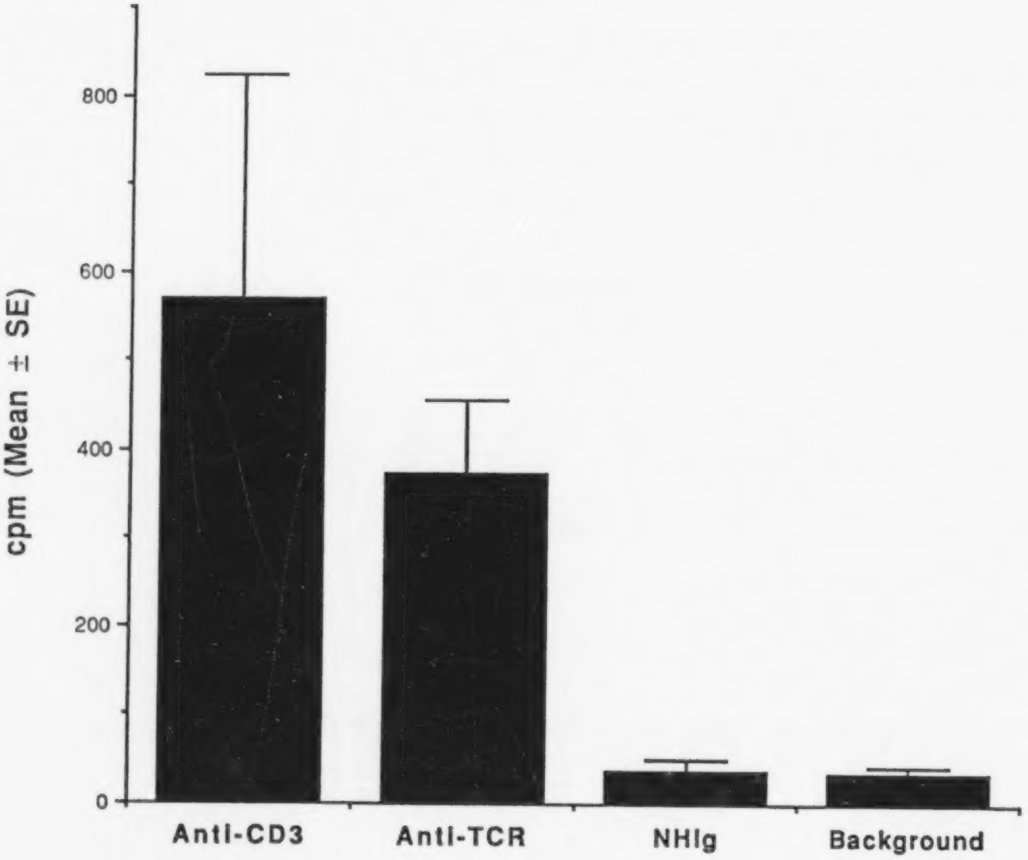


Fig. III.1. Specific binding of anti-CD3 and anti-TCR to thymocytes *in vivo*. Groups of 3 mice (4 week-old Balb/c) were intraperitonealy injected with ^{125}Iodinated protein A-purified anti-CD3ε (6.3 x 10^6 cpm), anti-TCRβ (5.4 x 10^6 cpm) or normal hamster immunoglobulins (6.1 x 10^6 cpm). Animals were terminated at 6 h post injection. Thymuses were removed, made into single cell suspensions and washed. The amount of bound antibodies of the washed thymocytes were counted with a gamma counter. NHIg: normal hamster immunoglobulin.

Table III.2. Localization of Anti-CD3 Post Intraperitoneal and Intravenous Injection

	Thymus	Liver	Spleen	MLN [#]	Lung
i.p	2761 ± 599	106227 ± 6427	10681 ± 379	9395 ± 594	13636 ± 1068
i.v.	1936 ± 235	100453 ± 16241	9155 ± 1035	7737 ± 2523	15179 ± 2855

Purified anti-CD3 was labeled with ^{125}I and injected into mice either i.p. or i.v. Each group has 3 mices. Each mouse received 1 x 10^6 cpm of the labeled antibody. The amount of labeled antibody in the different organs were determined 6 hours post injection. Data are presented as mean ± SE.

[#] mesenteric lymph node.

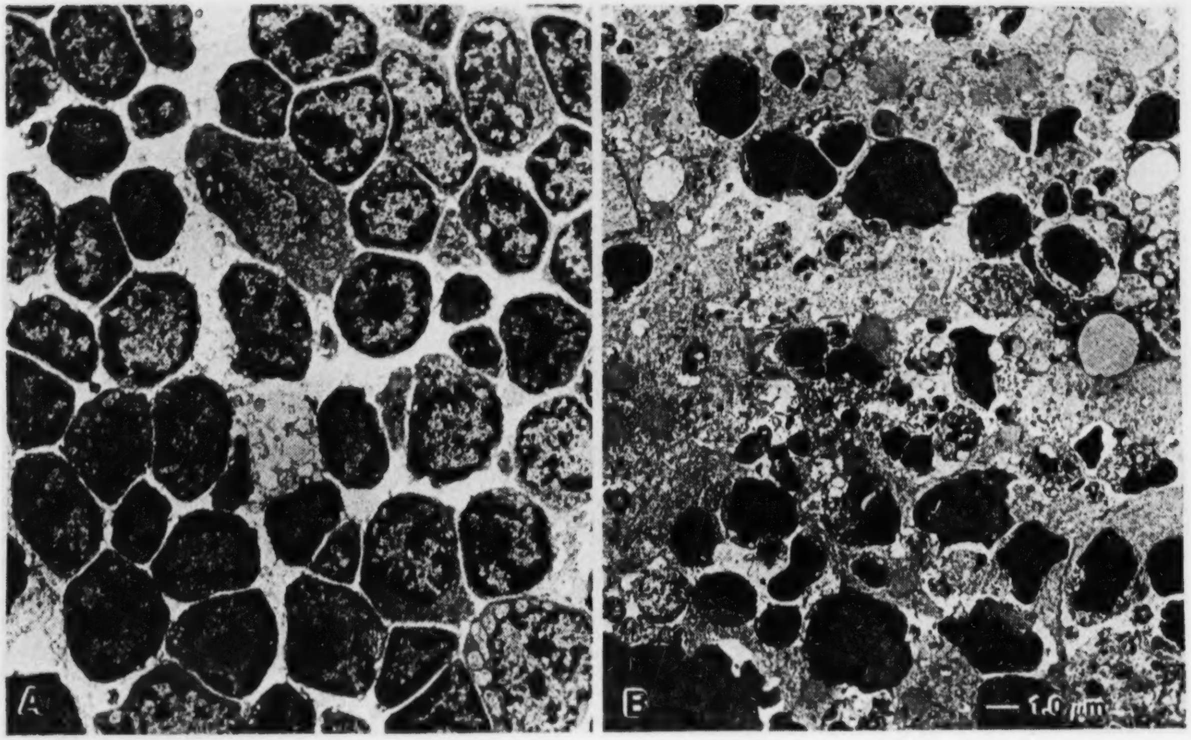


Fig. III.2. Transmission electromicrographs of the thymuses from a mouse treated with normal hamster serum (A) or with anti-CD3(145-2c11) (B). Three-week-old BALB/c mice were injected i.p. with 0.3 ml of 10 X diluted normal hamster serum or 0.3 ml of 50 X concentrated 145-2c11 culture supernatant (both containing ~30 µg of antibody). Thymuses were fixed at 12 h post treatment. The pictures are representatives of the three mice used for each treatment. (Magnification x1700).

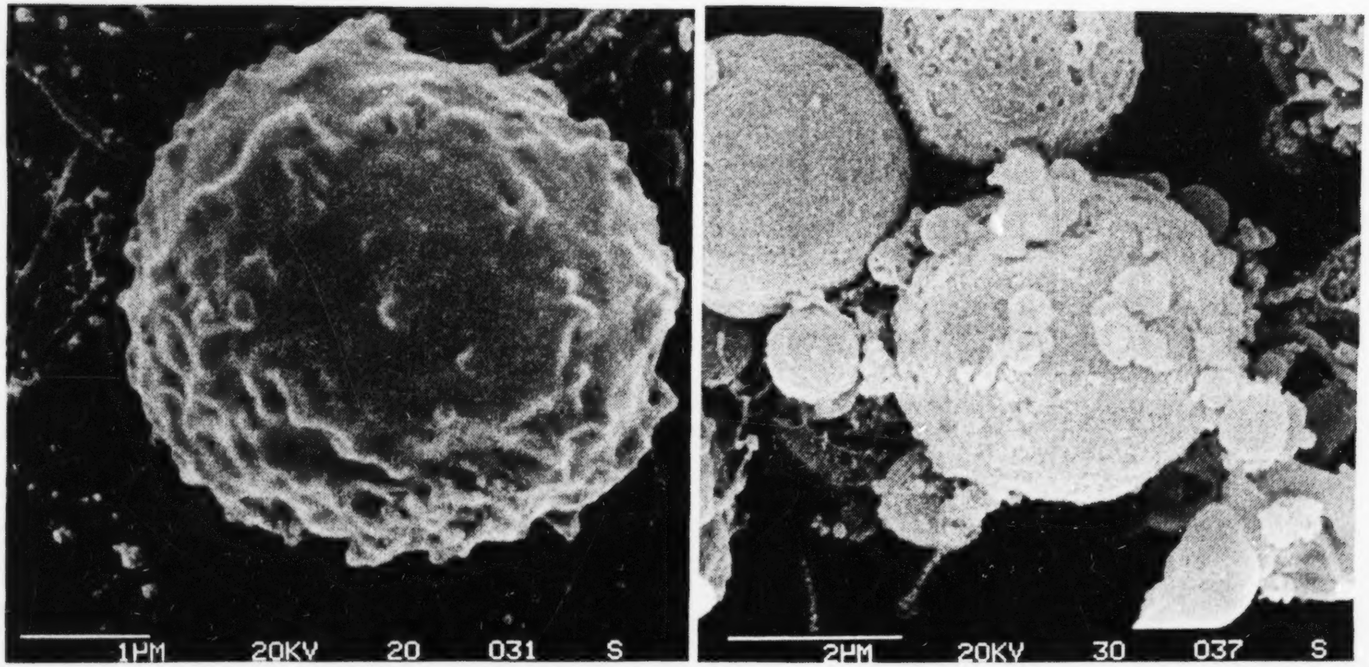

1PM 20KV 20 031 S
2PM 20KV 30 037 S

Fig. III.3. Scanning electronmicrographs of the thymus from mice injected i.p. with 0.3 ml of 10 X diluted normal hamster serum (A) or 0.3 ml of 50 X concentrated 145-2c11 culture supernatant (containing ~30 μg of antibody) (B). Thymuses were prepared from 4 week old Balb/c at 12 h post treatment.

examined this by *in vivo* treatment of mice with normal hamster serum or anti-CD3, and analyzed genomic DNA extracted from thymuses, spleens and lymph nodes on a 1% agarose gel. There was dramatic DNA fragmentation in the DNA samples from anti-CD3-treated thymus, but DNA samples from control antibody-treated thymuses or anti-CD3-treated spleens or lymph nodes did not migrate far from the loading wells into the gel, indicating that the DNA in these samples was not fragmented (Fig. III.4). To identify the thymic location of cell death induced by activation, thymus paraffin sections were stained with hematoxylin and eosin. It was observed on the histological sections that anti-CD3-induced cell death was localized in the cortex of the thymus (Fig. III.5B), where immature thymocytes are found (Sprent, 1989). Cell death was not observed in the medulla (Fig. III.5A) where mature thymocytes reside, nor in the peripheral lymphoid organs from anti-CD3-treated animals (Fig. III.5C). Thus, we concluded that activation through the T cell receptor complex induces apoptosis in immature thymocytes.

C. *In vivo* Administration of Superantigen Induces Cell Death in the Thymus

A possible alternative explanation for the effects of *in vivo* administration of anti-CD3 (or anti-TCR) is that these antibodies induce the production of a soluble factor toxic to thymocytes. This could be ruled out by using a more specific stimulus which would predictably affect only one subpopulation of the total thymocytes at a particular stage of thymocyte development. Bacterial enterotoxin superantigens represent one such stimulus. An example is staphalococcal enterotoxin B (SEB), which has been shown to preferentially stimulate T cells bearing TCR Vβ3 or Vβ8 (White et al., 1989). We injected SEB into Balb/c mice to determine whether this specific activator could induce cell death in the thymus. Sixteen hours post SEB injection, thymocytes were isolated and lysed with a nonionic detergent-containing buffer. After centrifugation, the fragmented DNA was separated from intact DNA. Fig. III.6 depicts that the resulting fragmented DNA in the

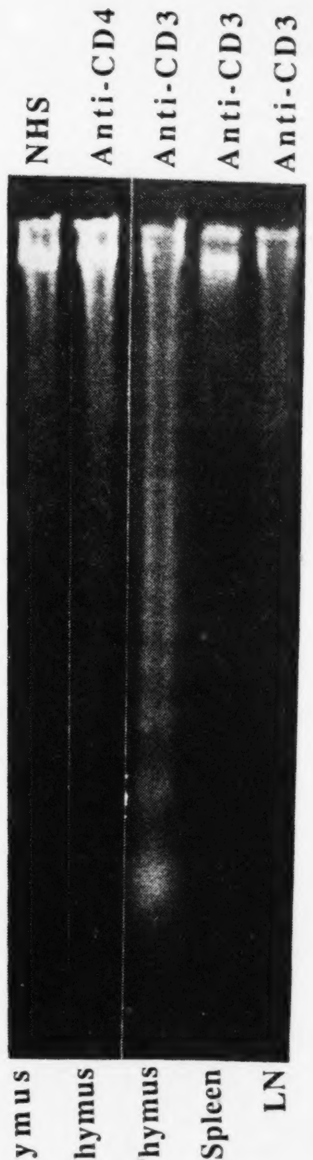

NHS
Anti-CD4
Anti-CD3
Anti-CD3
Anti-CD3
Thymus
Thymus
Thymus
Spleen
LN

Fig. III.4. Anti-CD3 induced DNA fragmentation in the thymus. Three-week-old BALB/c mice were injected with 0.2 ml of $(NH_4)_2SO_4$-precipitated normal hamster serum, or anti-mouse CD4 (GK1.5), or anti-mouse CD3 (145-2c11). Total genomic DNA was extracted from thymus, spleen, or lymph node 18 h after injection. Only anti-CD3 treated thymus showed DNA fragmentation on agarose gel. LN: mesenteric lymph node. NHS: normal hamster serum.

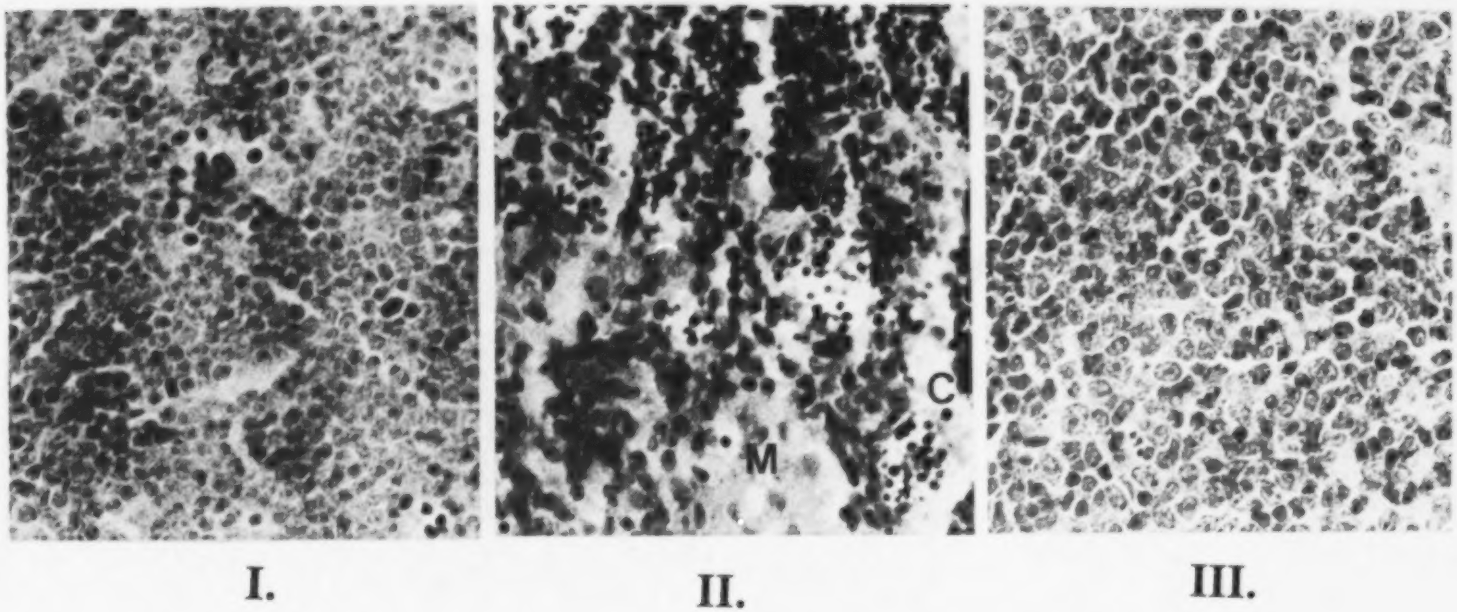


Fig. III.5. Anti-CD3 induced cortical thymocyte death. Mice were treated with $(NH_4)_2SO_4$-precipitated 145-2c11 culture supernatant (containing ~30 µg of anti-CD3, B &C). Control mice were treated with normal hamster serum (I). Thymus (I & II) and spleen (III) were removed and fixed 14 h after anti-CD3 injection. Paraffin sections were stained with hematoxylin and eosin. Numerous dead cells which are darkly stained appeared in the cortex (C) of the thymus treated with anti-CD3. (Magnification X 400). M: medulla.

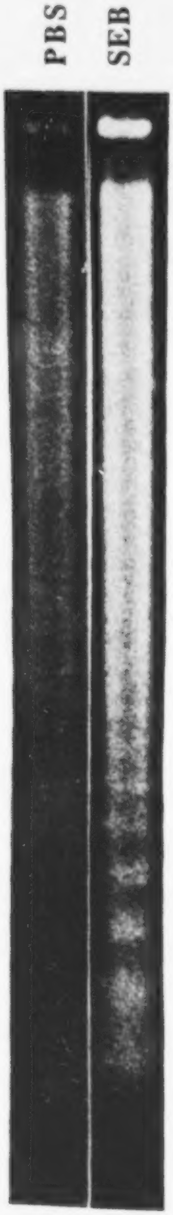


Fig. III. 6. DNA fragmentation in thymocytes induced by SEB *in vivo*. Four-week-old BALB/c mice were injected i.p. with 200 µg of SEB in PBS or PBS alone. Fragmented DNA was isolated 12 hours after treatments, and run on gel agarose gel.

supernatant of cell lysates from thymuses of SEB treated mice contained significant amount of fragmented DNA, as compared with control thymuses. *In vivo* administration of SEB is known to deplete only those immature thymocytes that bear Vβ3 or Vβ8 TCRs (White et al., 1989). Recently, Jenkinson and colleagues (1989) have shown that exposure of developing thymocytes to SEB in fetal thymus organ culture leads to activation-induced apoptosis in those thymocytes bearing the appropriate TCRs. Taken together with our results, it is demonstrated that the exposure of developing thymocytes to SEB *in vivo* leads to apoptosis in specific thymocyte subpopulations, implying that activation induced cell death in thymocytes is a specific process of activated thymocytes.

D. Anti-CD3 *in vivo* Induces Cell Death in $CD4^+CD8^+$ and a Subset of $CD4^+CD8^-$ Thymocytes.

Cells undergoing apoptosis experience cell surface glycoprotein changes; they can be then recognized by macrophages and subsequently removed (Duvall et al., 1985). Hence, if activation of immature thymocytes *in vivo* by anti-CD3 induces apoptosis, an obvious decrease in the number of thymocytes should be seen, provided that the rate of thymocyte regeneration does not exceed that of their death. T cell surface CD4 and CD8 molecules have been used to define the maturity of thymocytes (Scollay, 1991). These markers have also been adopted in this study to examine the population dynamics of thymocytes at 20 and 40 h after anti-CD3 treatment. FacScan analysis using phycoerythrin (PE) labeled anti-CD4 and fluorescein isothiocyanate (FITC) conjugated anti-CD8 antibodies revealed a dramatic loss in the $CD4^+CD8^+$ double positive thymocyte subpopulation at 40 h post anti-CD3 treatment as compared with normal hamster serum treated thymus (Fig. III.7). However, this analysis does not give much information on the fate of the double positive cells, that is, whether they were removed by phagocytes or underwent differentiation. To solve this problem the total cell number of each thymus tested was determined, then the number of cells in each population was calculated according to the

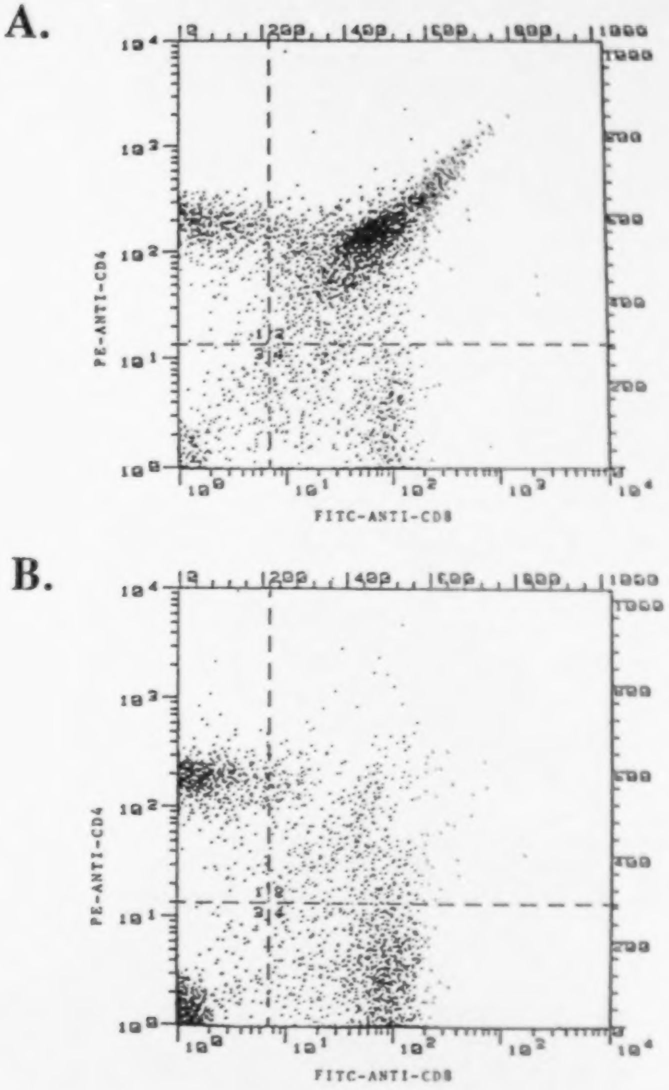


Fig. III. 7. FacScan analysis of the changes in the thymocyte populations after anti-CD3 administration. BALB/c mice were treated with normal hamster serum (A), or anti-CD3 (B). Thymocytes were harvested 40 h later and stained with FITC-labeled anti-CD8 and PE labeled anti-CD4.

percentage determined by FacScan (Table III.3). The dominant effect of anti-CD3 injection was the elimination of double positive cells by 40 h post injection. The absolute number of $CD4^{+}CD8^{-}$ cells was also significantly reduced (Table III.3) ($p<0.05$ at 20 h and $p<0.001$ at 40 h, as compared with control mice). There was no significant change in the absolute cell number in the double negative or the $CD4^{-}CD8^{+}$ cell populations.

Guidos et al. (1990) have shown that there are three populations of thymocytes according to the level of expression of αβTCR on the cell surface, one population is $\alpha\beta TCR^{negative}$, another is $\alpha\beta TCR^{low}$ and the third is $\alpha\beta TCR^{high}$. When we analyzed the level of CD3 expression (indicating the level of TCR on the cell surface), thymocytes could be distinguished into four populations: $CD3^{negative}$, $CD3^{low}$, $CD3^{intermediate}$ and $CD3^{high}$. It has been demonstrated that the majority of double positive cells are $CD3^{low}$ (Finkel et al., 1989). Fig. III.8 shows that cell depletion induced by *in vivo* administration of anti-CD3 was dominantly in $CD3^{low}$ and $CD3^{intermediate}$ populations.

E. Differences in Cell Death Induced by Anti-CD3 and Anti-TCR

Finkel et al. (1990) using an organ culture system, found that anti-TCR only induced depletion of a subset of thymocytes induced by anti-CD3. The present study was intended to determine the differential effect of anti-TCR and anti-CD3 on depletion of thymocytes *in vivo*. Equal amounts of each antibody was injected into mice before examination 40 h later. The absolute number of cells in each of the different thymocyte populations was calculated by multiplying the total thymocyte number with the percentage indicated by FacScan (Table III.4). Anti-CD3 induced depletion of most of the double positive cells and some of the $CD4^{+}CD8^{-}$ cells, as observed previously. Anti-TCR induced less depletion of double positive cells compared with that by anti-CD3. In addition, anti-TCR did not induce depletion of any other population of thymocytes.

Table III.3. Effects of *In Vivo* Administration of Anti-CD3 Antibody on Thymocyte Subpopulations[#]

	CD4$^-$CD8$^-$	CD4$^+$CD8$^-$	CD4$^-$CD8$^+$	CD4$^+$CD8$^+$
	(numbers shown are millions of cells/thymus, mean ± SE, n = 4 for all groups)			
NHS Control	18.8 ± 2.0	28.3 ± 2.1	8.25 ± 0.8	135.0 ± 11.7
20 hrs post anti-CD3	15.5 ± 1.9	17.4 ± 1.2	5.95 ± 0.2	12.3 ± 2.6
p (vs control)	$p>0.1$	$p<0.05$*	$p>0.08$	$p<0.01$*
40 hrs post anti-CD3	18.2 ± 4.2	11.1 ± 2.1	8.5 ± 1.5	1.5 ± 0.23
p (vs control)	$p>0.1$	$p<0.01$*	$p>0.1$	$p<0.01$*

[#] Groups of 4 animals were injected with normal hamster serum (NHS) or anti-CD3 antibodies (145-2c11). Twenty or 40 hours post injection, each thymus was removed, thymocytes counted, and stained with FITC-anti-CD8 and PE-anti-CD4. Staining are assessed by FacScan. The percentage of each population were converrted to absolute cell numbers based on total thymocyte counts for each animal (* significant difference based on ANOVA).

Table III.4. Differential Effects of *In Vivo* Administration of Anti-CD3 and Anti-TCR Antibodies on Thymocyte Subpopulations

(numbers shown are millions of cells/thymus, mean ± SE, n = 5 for NHS and anti-CD3; 4 for group anti-TCR)

	Total	$CD4^-CD8^-$	$CD4^+CD8^-$	$CD4^-CD8^+$	$CD4^+CD8^+$
NHS Control	200.75 ± 11	18.1± 3.4	27.0 ± 2.8	15.4 ± 2.6	138.5 ± 4.2
anti-CD3	42.5 ± 4.2	17.0 ± 3.0	11.5 ± 0.9	9.2 ± 0.8	3.1 ± 0.2
p (vs control)	$p<0.01$*	$p>0.1$	$p<0.01$*	$p>0.1$	$p<0.01$*
anti-TCRβ	85.7 ± 9.5	23.7 ± 5.1	18.2 ± 2.2	16.5 ± 1.4	27.2 ± 5.9
p (vs control)	$p<0.01$*	$p>0.1$	$p>0.06$	$p>0.1$	$p<0.01$*
p (vs anti-CD3)	$p<0.02$*	$p>0.1$	$p>0.1$	$p>0.1$	$p<0.03$*

Groups of 4-5 animals were injected with normal hamster serum (NHS, n=5) or anti-CD3 antibodies (145-2c.11, n=5) or anti-TCR (H57-957). Forty hours post-injection, each thymus was removed, thymocytes counted, and stained with FITC-anti-CD8 and PE-anti-CD4. Staining was assessed by FacScan. The percentage of each population was converted to absolute numbers based on total thymocyte counts for each animal (* significant difference based on ANOVA).

To examine the differential effects of anti-CD3 and anti-TCR on the depletion of thymocytes on the basis of the level of CD3 expression, similar analyses were performed as in Fig. III.7. As compared with the treatment with anti-CD3, treatment with anti-TCR mainly depleted cells expressing low levels of CD3 (Fig. III.8 and Table III.5). However, differential ability of the two antibodies to induce apoptosis in the thymus could be due to the differential effect of the two antibodies on activating T cells. We, therefore, examined the relative abilities of anti-CD3 and anti-TCR antibodies to induce apoptosis in T cell hybridomas. Each of the two antibodies were coated onto two 96-well tissue culture plastic plates with doubling dilution. One of the plates was assessed for the amount of antibodies by ELISA. The second plate was used to activate T cell hybridoma A1.1 cells, which undergoes activation induced cell death. As shown in Fig. III.9, the same amount of both antibodies showed the same level of activity on T cell hybridomas (Fig. III.9) (Further discussion on activation induced cell death in T cell hybridomas will be followed in Chapter IV).

The above experiments have thus shown that polyclonal activation of immature thymocytes through the TCR complex *in vivo* leads to their depletion. The differential effects of anti-CD3 and anti-TCR suggest the importance of the regulation or coupling of TCR and CD3 in the induction of apoptosis. Under most circumstances, activation proceeds via binding of TCR (versus CD3) and therefore, it is most likely the anti-TCR-responsive population ($CD4^+CD8^+$) that is most susceptible to antigen-induced apoptosis.

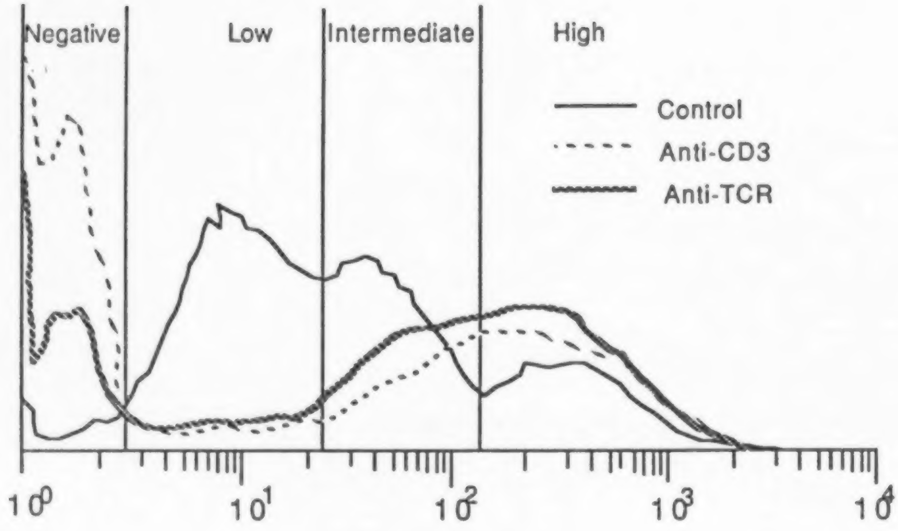


Fig. III.8. The levels of CD3 expression on thymocytes after *in vivo* anti-CD3 or anti-TCR treatment. Forty hours post antibody treatment, thymocytes were harvested, stained with anti-CD3 antibodies, followed by FITC-labeled rabbit anti-hamster Ig and analyzed by FacScan.

Table III.5. Changes in Thymocyte Subpopulation as Indicated by the Levels of Surface CD3 after Injection of Anti-CD3 or Anti-TCR Antibody.

(numbers shown are millions of cells/thymus, mean ± SE, n = 5 for NHS and ANTI-CD3; 4 for ANTI-TCR)

	CD3$^-$	CD3^{lo}	CD3^{int}	CD3^{hi}
NHS Control	15.7 ± 0.82	137.2 ±13.2	65.4 ± 3.9	58.1 ±1.2
anti-CD3	14.4 ± 1.5	2.6 ± 0.7	6.9 ± 0.9	13.2 ± 2.1
p (vs control)	p=0.593	p=0.0005*	p=0.0003*	p=0.001*
anti-TCRβ	16.4 ± 2.1	7.6 ± 1.7	26.0 ± 1.8	26.4 ± 2.8
p (vs control)	p=0.856	p=0.0042*	p=0.0073*	p=0.002*
p (vs anti-CD3)	p=0.190	p=0.152	p=0.0004*	p=0.0047*

Groups of 4-5 animals were injected with normal hamster serum (NHS, n=5) or anti-CD3 antibodies (145-2c.11, n=5) or anti-TCR (H57-597, n=4). Forty hours post injection, each thymus was removed. The thymocytes counted, and stained with anti-CD3 followed by FITC-anti-hamster Ig. Stained thymocytes were assessed by FacScan. The percentage of each population was convertted to absolute cell numbers based on total thymocyte counts for each animal (* significant difference based on ANOVA).

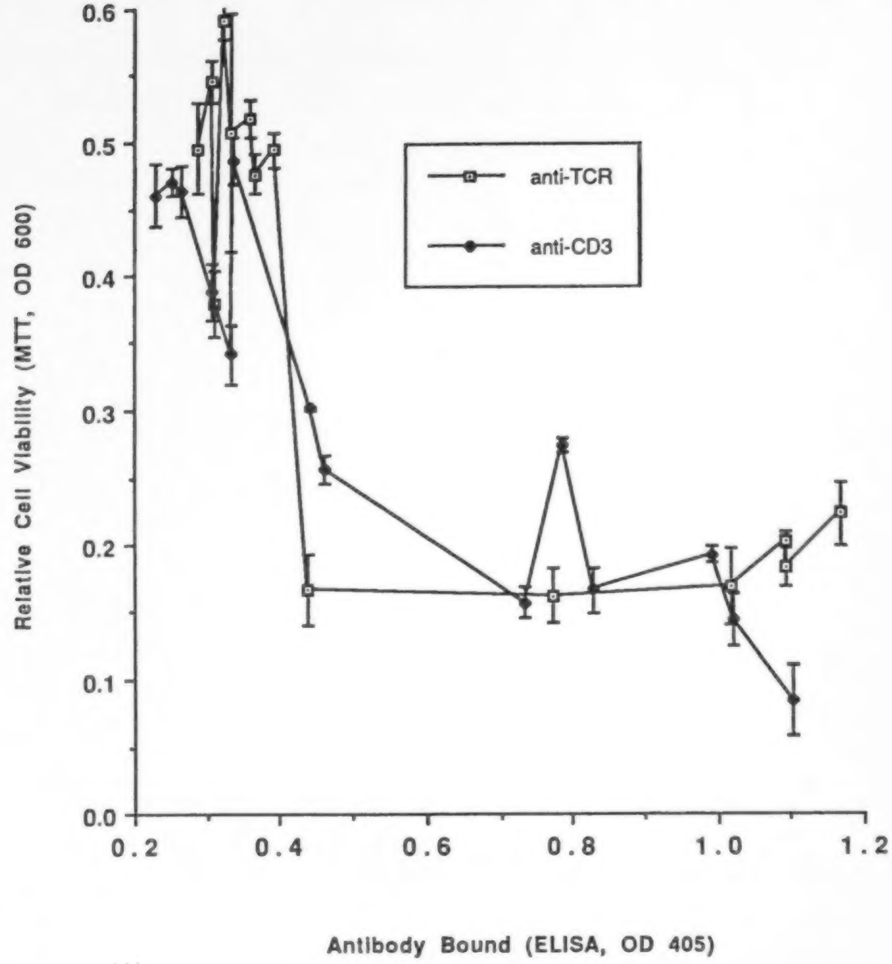


Fig. III.9. Comparison of the ability of anti-CD3 and anti-TCR antibodies to induce apoptosis in T cell hybridoma A1.1. Two sets of 96-well plates were coated with doubling dilutions of antibodies. One set was analyzed by ELISA to determine the relative amount of antibody. The other one used to determine the ability of antibodies to stimulate a T cell hybridoma, A1.1, to undergo apoptosis. Cell viability was determined by MTT assay at 24 h post stimulation with the antibodies. The relationship between the amount of antibody and the ability to induce cell death is shown.

IV. ACTIVATION INDUCED CELL DEATH IN T CELL HYBRIDOMAS *IN VITRO*

In vivo administration of antibodies to the T cell antigen receptor complex demonstrated that activation of immature thymocytes caused them to enter a suicide pathway, which we proposed to be the mechanism of negative selection of autoreactive T cells during T cell development. To further characterize the cellular and molecular bases for this phenomenon, it is advantageous to have an *in vitro* model system. However, thymocytes are highly unstable in *in vitro* culture, a large proportion of $CD4^+CD8^+$ thymocytes tend to die shortly after *in vitro* culture (Swat et al., 1991). In addition, thymocytes *in vivo* constitute a mixture of cells of different types and at different stages of development. Thus an *in vitro* system with a homogeneous cell population would be immensely useful for the characterization of the mechanism of apoptosis in general, and activation induced cell death in particular. Ashwell et al. (1987) reported that some T cell hybridomas cease to incorporate tritiated thymidine after activation, but it was not clear whether this was due to inhibition of cell division or due to death of these cells. The following experiments were set up to determine whether this was actually a process of activation induced apoptosis.

A. Activation of T Cell Hybridoma A1.1 Results in Cell Death

A1.1 is a T cell hybridoma produced by fusion of a poly-18-specific, $CD4^+TCR\alpha\beta^+$ T cell with a thymoma, BW5147 (Fotedar et al., 1985). Upon activation with the antigenic peptide plus $I\text{-}A^d$ bearing antigen presenting cells, or immobilized anti-CD3, or other mitogenic stimulators, A1.1 cells lost their ability to exclude Trypan blue (Fig. IV.1). At the same time these activated cells released lymphokines, as detected by the ability to stimulate the growth of an IL-2 dependent cell line, CTL.L cells (Fig. IV.2). The property of A1.1 cells to undergo cell death after activation might come from the fusion partner, thymoma BW5147, because BW5147

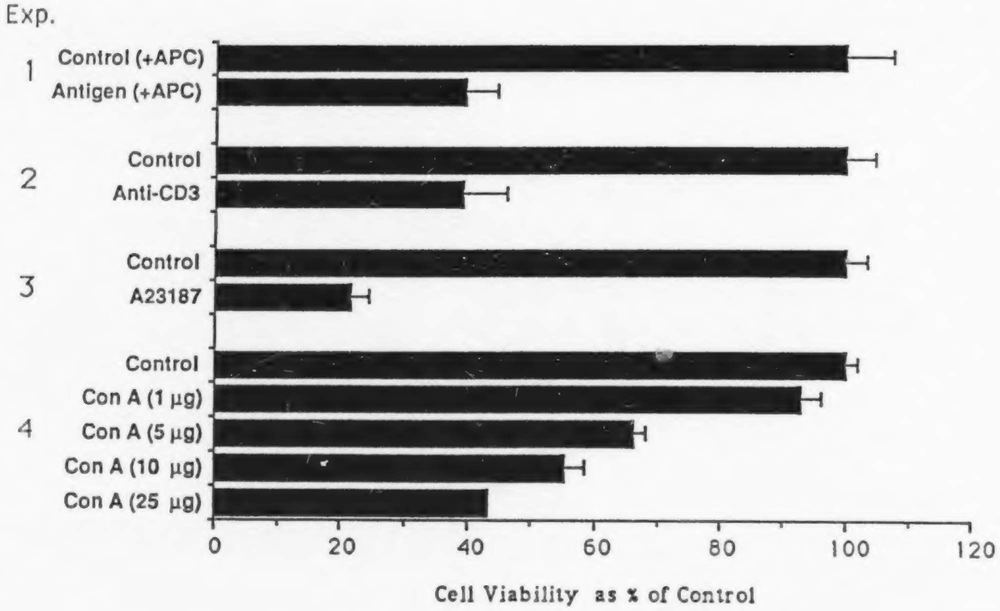


Fig. IV.1. Activation of T cell hybridoma A1.1 results in reduction in cell viability. A1.1 cells (1 x 10^5) were cultured in 96-well plates in 200 µl of RPMI supplemented with 5% fetal calf serum in the presence of antigen plus APC [5 µg poly18 (optimal dose as determined by a titration experiment) plus 2 x 10^4 irradiated TA3 cells], A23187 (125 ng/ml), ConA (concentration as shown), or anti-CD3 coated plastic plate. At 24 h post activation cell viability was determined by Tryoan blue exclusion. Data ploted are from 4 different experiments and presented as a percentage of control.

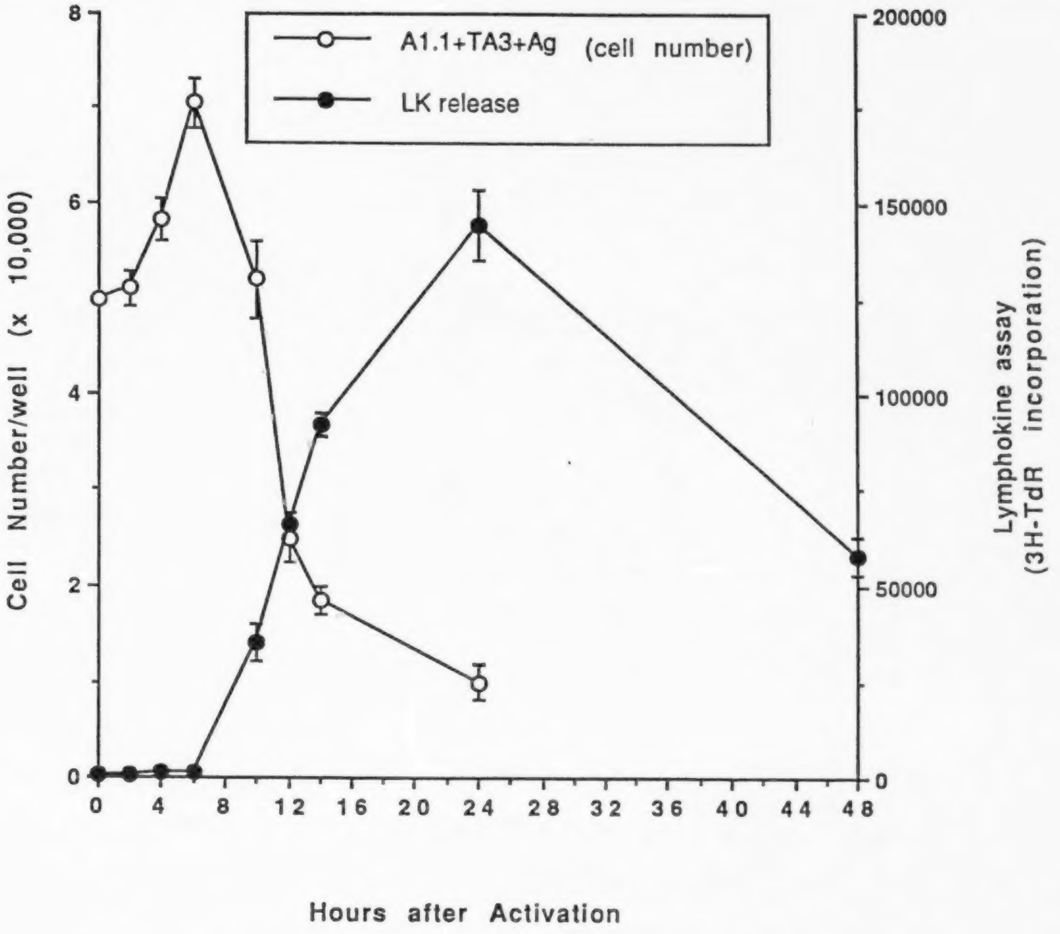


Fig. IV.2. The kinetics of activation induced cell death and lymphokine production in T cell hybridoma A1.1 cells. Cells (1 X 10^5) were cultured in each well of a 96-well plate with 5 µg poly18 plus 2 x 10^4 TA3 cells. Cell viability was assayed by Trypan blue exclusion at different times post activation. The amount of lymphokine in the supernatant at different times post stimulation was detected by its ability to support the proliferation of CTL.L cells. Data are expressed as mean ± SE).

cells could be induced to reduce MTT (which is converted from a yellow solute to purple crystals by active mitochondria of living cells, but not that of dead cells; Mosmann, 1983) undergo cell death and lymphokine production with Con A or ionophore A23187 (Fig. IV.3), which possibly represents a particular thymocyte developmental stage during which activation leads to cell death, and thus mimics activation induced immature thymocyte death *in vivo*.

B. Morphological Changes in Activation Induced Cell Death in T Cell Hybridomas

Activation induced cell death in the T cell hybridoma is associated with dramatic morphological changes, which could be easily observed under a light microscope. Fig. IV.4.B shows the morphology of A1.1 cells under the light microscope. Nine hours after treatment with an anti-CD3 coated plastic tissue culture plate, the cell membranes were deformed, their cytoplasm became unevenly distributed and formation of apoptotic bodies (ab), which is a portion of cytosolic structure surrounded by intact cell membrane (Fig. IV4.A).

To examine the ultrastructural changes in activation induced cell death in T cell hybridomas, A1.1 cells were treated with antigen plus antigen presenting cells, or immobilized anti-CD3. At different times post activation cells were prepared for transmission or scanning electromicroscopy. As a comparison, A1.1 cells were also treated with 0.1% sodium azide for 8 h, which inhibits the respiratory chain in a cell and subsequently induces necrosis. After activation with anti-CD3, nucleochromatin of the cells formed dense dark masses (Fig. IV.5 B,D,F), while the cytoplasmic structure was relatively maintained. Chromatin condensation is one characteristic of apoptosis, whereas cells undergoing necrosis induced by azide showed swollen nuclei and destruction of the cytoplasmic structure (Fig. IV.5.C,E). Activation induced cell death was also examined under the scanning

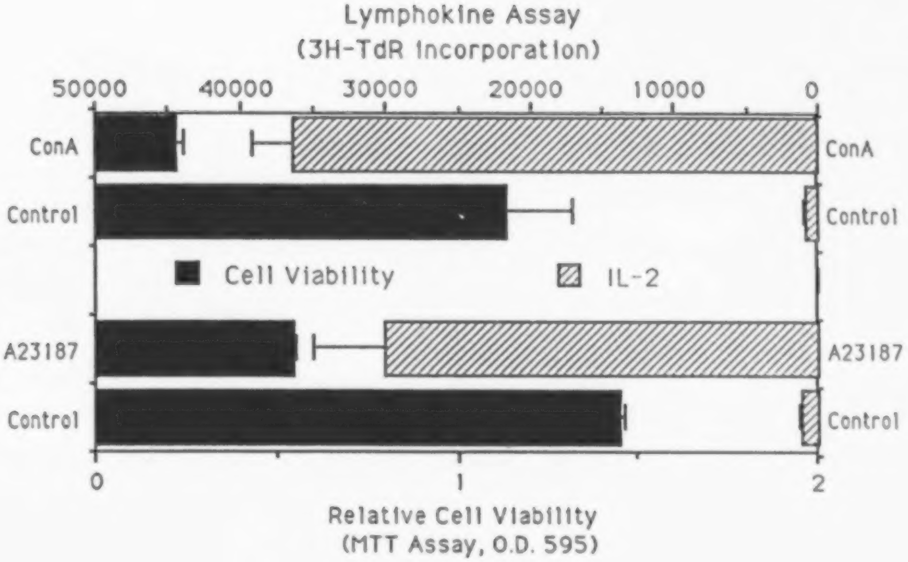


Fig. IV.3. Activation induced cell death and lymphokine production in thymoma BW5147 cells. Cells (1 x 10^5) were cultured in a 96-well plate in 200 μl of RPMI supplemented with 5% fetal calf serum (in the presence of 10 μg ConA or 125 ng/ml A23187). Cell viability was determined by MTT at 24 h post activation. The amount of lymphokine present in the supernatant at different times post stimulation was detected by ability to support the proliferation of CTL.L cells.

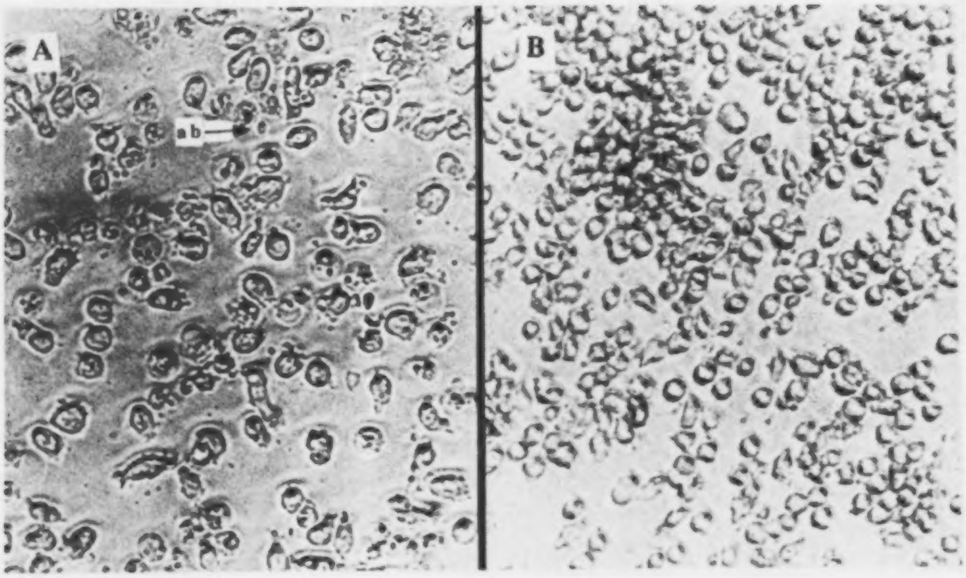


Fig. IV.4. Morphological changes in activation induced A1.1 cell death as viewed under a light microscope. A1.1 cells, treated with (A), or without (B), anti-CD3, were photographed using an inverted light microscope at 12 h after stimulation. ab: apoptotic body.

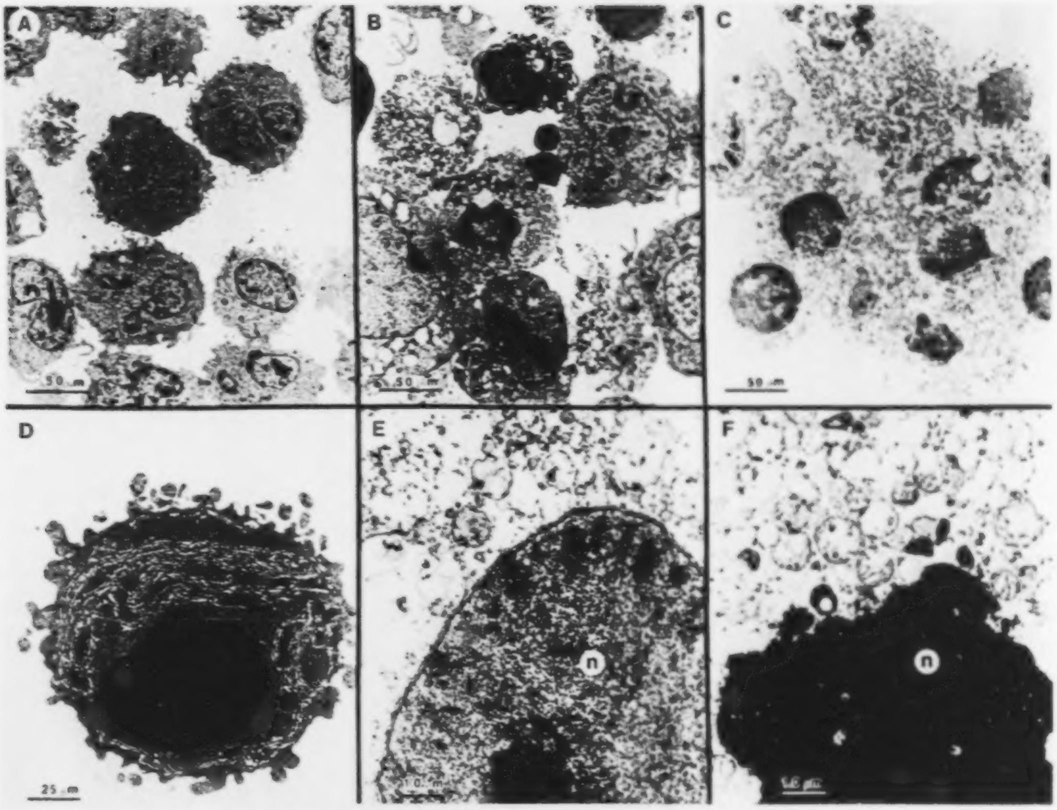
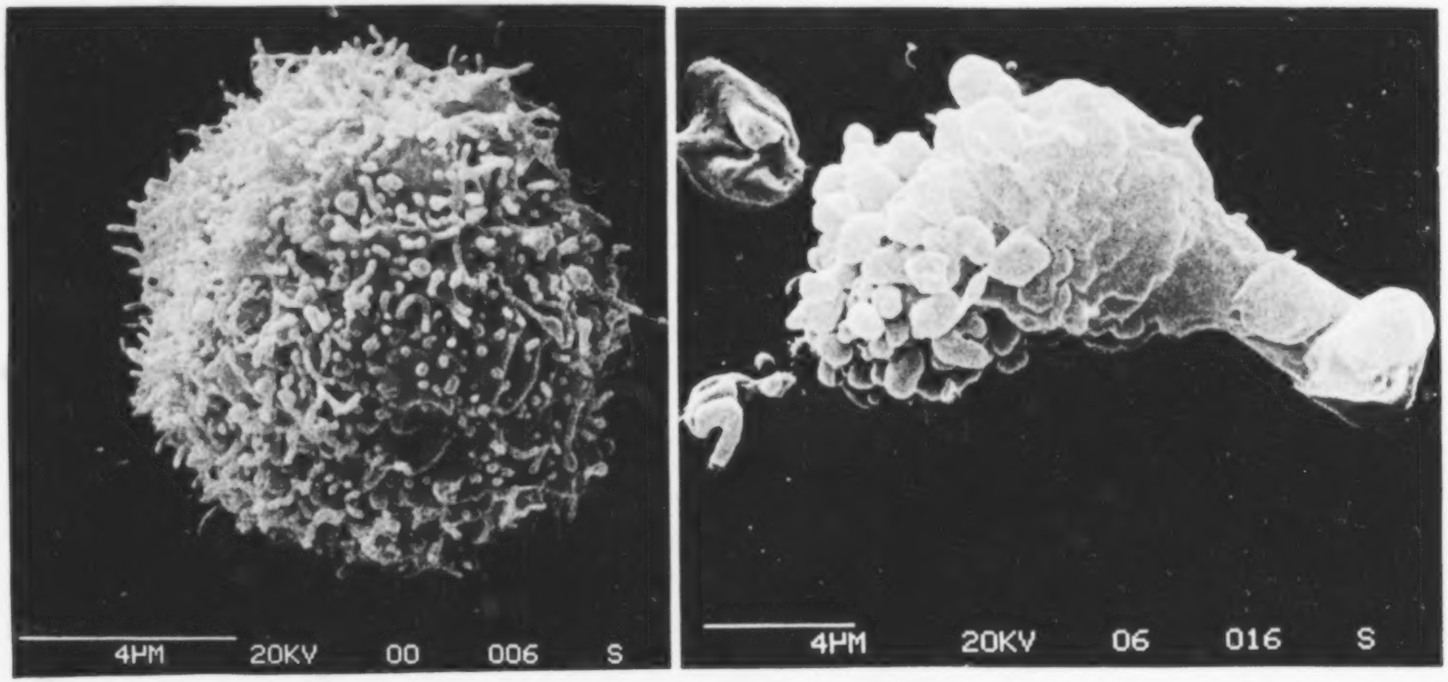


Fig. IV.5. Morphological changes of activation induced cell death vs. necrosis in T cell hybridoma A1.1 cells, viewed by transmission electron microscopy. A1.1 cells were treated with APC plus specific antigen, anti-CD3 or 0.01% sodium azide. Eight to 14 h after treatment, cells were prepared for transmission electron microscopy. A. Normal cells; B. Antigen plus APC for 8 h; C. Sodium azide for 14 h; D. Antigen and APC for 8 h; E. Sodium azide for 14 h; F. Plastic coated anti-CD3 for14 h. n, nucleus.



A.

B.

Fig. IV.6. Morphological changes associated with activation-induced cell death in T cell hybridoma A1.1 cells, viewed by the scanning electron microscopy. A1.1 cells were treated either with (B) or without (A) anti-CD3 for 6 h, attached to a microscope slide cover slip with poly-lysine and prepared for scanning electron microscopy.

electromicroscope. Unstimulated cells showed microvilli on their surface (Fig. IV.6A). After anti-CD3 stimulation, the microvilli disappeared and pedunculate protruberances developed on the cell surface (Fig. IV.6B).

C. Fragmentation of Genomic DNA Associated with Activation Induced Cell Death in T Cell Hybridoma

It is generally accepted that cell death through apoptosis results in degradation of genomic DNA into an approximately 200 base pair nucleosomal-sized ladder. We have demonstrated that activation induced immature thymocyte death *in vivo* showed this type of characteristic DNA fragmentation. To determine if such a phenomenon exists in activation induced cells in the T cell hybridoma, genomic DNA was isolated from A1.1 cells at different times after their treatment with anti-CD3 coated plastic. As shown in Fig IV.7, DNA fragmentation as detected in this way was apparent at approximately 5 h, and reached maximum at 7 h after activation.

D. "Commitment" to Apoptosis

Anti-CD3 alone in solution cannot activate T cell hybridomas. It must be coated onto plastic surface or be in combination with appropriate antigen presenting cells or cross linked by a secondary antibody (Mercep et al., 1988). This property was utilized to determine the kinetics of the commitment of T cell hybridoma to cell death after activation. A1.1 cells were incubated on an anti-CD3 coated plate. At different times cells were transferred to a plate precoated with serum and incubation was continued in tissue culture incubator for a total of 12 h. A quantitative assay of DNA fragmentation was used to determine the proportions of DNA fragmentation. This method uses Triton X-100, which is a non-ionic detergent and lyses cells but does not interfere with the nucleosome structure, thus allowing the separation of fragmented from non-fragmented DNA. Fig. IV.8B shows that at least 3-4 h of

Hours After activation
0 4 5 6 7 8

Fig. IV.7. DNA fragmentation associated with activation-induced cell death in T cell hybridomas. A1.1 cells were activated on anti-CD3 coated plastic tissue culture flasks. At various times after activation, cells were harvested and lysed with a lauryl sarcosinate containing buffer. After digestion with proteinase K and RNase A, DNA fragmentation was analysed on an agarose gel.

contact between anti-CD3 and A1.1 cells is needed to get a detectable amount of DNA fragmentation. PMA in combination with ionomycin can activate resting T cells and drive them to cell cycle and produce lymphokines. These are small molecules and their effect can be removed from cell culture by washing. We incubated A1.1 cells with PMA and ionomycin and washed with prewarmed medium at different times after activation and continued incubation for a total of 12 h. The relative cell viability was assayed by MTT. As shown in Fig. 8A, cell viability pattern was similar to that of DNA fragmentation after anti-CD3 stimulation for different times.

E. Requirement of Macromolecule Synthesis During Activation Induced Cell Death

Cycloheximide, an inhibitor of protein synthesis, and actinomycin D, which inhibits RNA synthesis, have been shown to interfere with *Xenopus* tail atrophy during metamorphosis (Weber, 1965; Tada, 1966), and to prevent thymocyte death induced by glucocorticoid (Wyllie et al., 1984). It is thus possible that macromolecule synthesis is also involved in activation induced cell death in T cell hybridomas. It should be pointed out that both actinomycin D and cycloheximide are toxic to cells, causing them to die approximately 15-20 h after treatment. We have already demonstrated that in *in vitro* culture, A1.1 cells start to die at 6-7 h after activation with anti-CD3. Cell death is maximal at 9-10 h. This system thus allows us to test the pharmacological effects of actinomycin D and cycloheximide during the early phase of cell death. We have not been able to detect cell death at early stages using the conventional Trypan blue exclusion method. We took advantage of the fact that living cells exclude fluorescein in the presence of protein, while dying cells, even at the early stages, do not. As shown in Fig. IV.9, at 8 h post activation with anti-CD3 antibody, the loss of cell viability in A1.1 cells, as assayed by the fluorescence method, was prevented by the presence of cycloheximide and actinomycin D in culture. In addition, when DNA

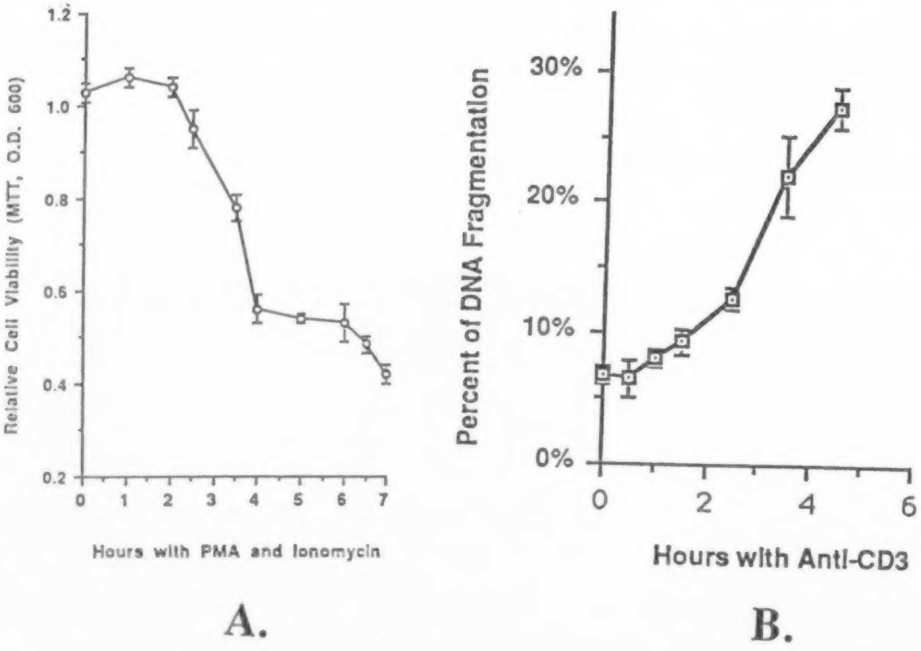


Fig. IV.8. The contact time between T cell hybridoma and the stimuli required for activation induced cell death in T cell hybridoma. (A) 1 x 10^5 A1.1 cells per well were cultured in a 96-well plate. Fifteen nM PMA plus 0.5 µM ionomycin were added at different times. Cells was then washed 3 times with 37 °C media, and 14 h after continuous culture, cell viability was assayed by MTT. Times indicated are the time of cells with PMA and ionomycin. (B) 1 x 10^5 ^{125}IUDR labeled A1.1 cells were incubated on an anti-CD3 precoated 96-well plate. At different times post stimulation, cells were transferred to a serum-coated plate. Twelve h after the initial stimulation, DNA fragmentation was assayed by measuring the radioactivity in the supernatant and in the sediment after lysing the cells with Triton X-100 and centrifugation and expressed as percentage of fragmentation.

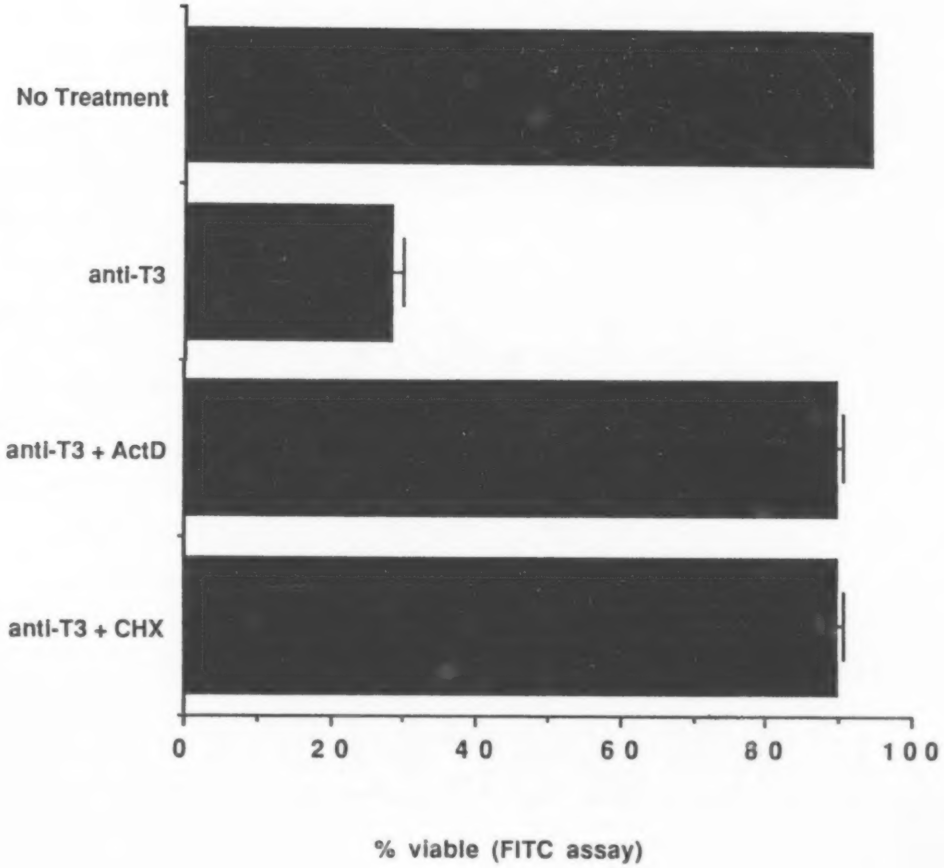


Fig. IV.9. Prevention of activation induced cell death by inhibition of macromolecule synthesis, as assessed by FITC exclusion assay. 10^5 A1.1 cells were cultured with 200 μl of RPMI supplemented with 5% FCS. Act D (5 μM) or CHX (50 μM) was added at 0 h and cell viability was assayed at 7 h post anti-CD3 stimulation.

was extracted at 12 h post stimulation, DNA fragmentation induced by activation in A1.1 cells was significantly reduced by actinomycin D and cycloheximide (Fig. IV.10). However, while both drugs profoundly reduced DNA fragmentation, neither completely prevented it. Possible explanations are the putative protein required for the fragmentation of genomic DNA may be already present in a small amount in cells, or inhibitors only give partial inhibition, or inhibitors themselves start to induce DNA fragmentation by whatever mechanism at 12 h post activation. Nevertheless, the observation that activation induced cell death in the T cell hybridoma was inhibited by cycloheximide or actinomycin D at an early stage after activation strongly suggests that activation induced cell death is an active suicide process.

We have, therefore, demonstrated that activation of T cell hybridoma results in cell death, which is an active process characterized by condensation of nucleochromatin, membrane blebbings and fragmentation of genomic DNA. The conclusion we draw is that cell death induced in the T cell hybridoma by activation is apoptosis.

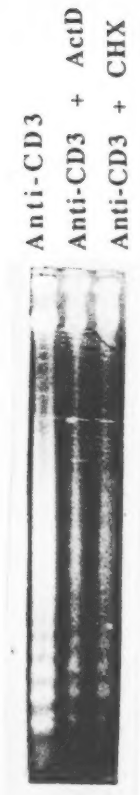

Anti-CD3
Anti-CD3 + ActD
Anti-CD3 + CHX

Fig. IV.10. Cycloheximide and actinomycin D inhibit DNA fragmentation during activation induced cell death in a T cell hybridoma. A1.1 cells were activated by anti-CD3 with/without actinomycin D (5 μM) or cycloheximide (50 μM) and DNA was extracted after 12 h in culture.

V. ACTIVATION INDUCED CELL DEATH IS A CELL-AUTONOMOUS EVENT

In the last two chapters, we have demonstrated that activation of T cell hybridomas, like that of immature thymocytes *in vivo*, results in apoptotic cell death. How do the activation signals through the T cell receptor result in apoptosis? There are three possible mechanisms that account for the apoptosis event in such cells. The first possibility is that activation of these cells leads to the production of soluble "death factors" that bind to cells and kill them. Alternatively, activation through the T cell receptors results in two distinct events: the production of "death factors", and the expression of cell surface receptors for those factors. The binding of these "death factors" to their novel receptors causes the death of activated cells. A third possibility is that death of activated cells is strictly a result of an autonomous, probably intracellular process. The following experiments will test these possibilities.

A. The Role of Extracellular Mediators in Activation Induced Cell Death in T Cell Hybridomas (The First Possibility)

To determine the role of extracellular mediators released by activated cells in induction of apoptosis, we first assayed the supernatant of anti-CD3 stimulated A1.1 cells for its ability to kill unactivated cells. We collected supernatant from A1.1 cell culture (10^5 cells/200 µl) at 6 h after activation with anti-CD3 coated plastic tissue culture plates. A1.1 cells (without activation) were cultured with the supernatant at concentrations of 0.78% to 50% prepared by two fold dilution. Cell viability was determined at 24 h after culture. No death of A1.1 cells was observed (Table V.1). A second experiment was performed to confirm this result. This time we employed two related T cell hybridomas, A1.1 and B1.1. B1.1 is another poly18 and I-A^d specific T cell hybridoma made by Fotedar et al. (1985). B1.1 recognizes $(EYA)_5$, a poly18 derivative, which is not recognized by A1.1. When ^{51}Cr labeled B1.1 was stimulated with $(EYA)_5$ plus antigen presenting cells (APC), nearly all the ^{51}Cr

Table V.1. The inability of TNF-α, TNF-β and anti-CD3 stimulated A1.1 culture supernatant to induce death of A1.1 cells.

Treatments	Concentrations	Cell Death
Anti-CD3	-	Yes
TNF-α	0.64 - 10 ng/ml	No
TNF-β	0.128 - 2 ng/ml	No
Supernatant*	0.78% - 50%	No

* Anti-CD3 activated A1.1 supernatants were collected at 6 hours post stimulation.

was released from the cells, while release was not significant when ^{51}Cr labeled A1.1 was incubated with the same antigen and APC. When ^{51}Cr labeled A1.1 cells were mixed with unlabeled B1.1 and incubated with $(EYA)_5$ plus APC, there was no induction of ^{51}Cr release (Table V.2), supporting the above conclusion that activation induced extracellular factors do not exert a bystander effect on neighboring cells.

B. The Role of the Expression of Receptors for Extracellular Mediators In Activation Induced cell death (The Second Possibility)

The inability of unactivated cells to respond to extracellular mediators in these experiments may be due to the lack of receptors for the extracellular mediators on unactivated cells, i.e., the second mechanism may operate. One possible candidate of the extracellular mediator of activation induced cell death in the T cell hybridoma is tumor necrosis factors (TNF), which have been shown to induce cell death in tumor cells and fibroblast cells (Larrick and Wright, 1990). We asked whether TNF could induce cell death in A1.1 cells. A1.1 cells were cultured with TNF-α (0.64—10 ng/ml) or lymphotoxin (TNF-β) (0.128—2 ng/ml) for 24 h. No cell death occurred (Table V.1). This indicates that TNF might not be the mediator of activation induced cell death. However, when employing a sensitive bioassay, the L-929 fibroblast system (Branch et al, 1991), we found that the supernatant of anti-CD3 activated A1.1 cells (5 x 10^5 cells/ml) contains about 200 pg/ml (approximately 40 IU/ml) of TNF, whereas TNF was not detectable in the supernatant of unstimulated A1.1 cell culture (Table V.3). Thus, activation induced cell death of A1.1 cells is accompanied by the production of TNF. The inability of TNF to kill unactivated cells may therefore possibly be due to the lack of TNF receptors on unactivated cells. Our next experiment clearly demonstrated that this is not the case. As many as 1,000 neutralizing units/ml of rabbit antiserum against murine TNF (Branch et al., 1991) applied in anti-CD3 activated A1.1 culture did not prevent activation induced cell death in the T cell hybridoma (Fig. V.1). Thus, even though TNF

Table V.2. Activation Induced Cell Death in T Cell Hybridoma is a Direct Effect of Activation Stimulus.

^{51}Cr -labeled Labeled*	Non-Labeled	^{51}Cr Release % of Total
A1.1	—	24.1%
B1.1	—	98.1%
A1.1	B1.1	15.7%
B1.1	A1.1	64.2%

* Total Cr^{51} release was measured by lysing labeled cells with 0.1% Triton X-100.

I-A^d bearing antigen presenting cell, B cell lymphoma TA3 and B1.1 specific antigen $(EYA)_5$ were included in every group.

Table V.3. The amount of TNF in the supernatant of anti-CD3 activated A1.1 cells*

Anti-CD3	Amount of TNF (pg)
—	NDt
+	210.3 ± 27

*5 X10^5 A1.1 cells were cultured with or without anti-CD3 for 7 hours, then supernatants were collected for TNF assay with the L929 fibroblast bioassay system.
NDt: Not detectable.

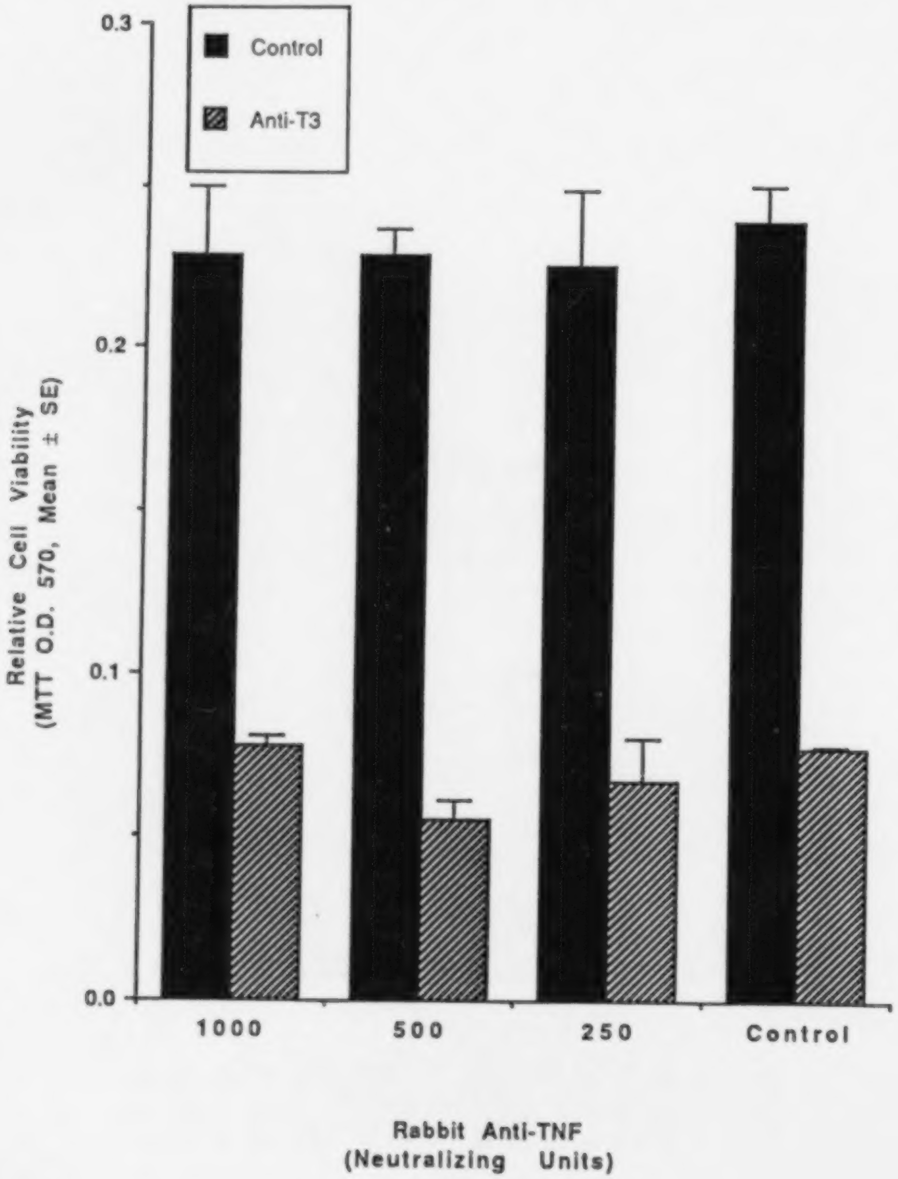


Fig. V.1. The inability of anti-TNF antibody to inhibit anti-CD3 induced cell death in T cell hybridomas. A1.1 cells were treated with anti-CD3 in the presence of different concentrations of rabbit anti-mouse TNF. Cell viability was determined at 10 h by MTT.

is produced by activated T hybridoma A1.1 cells, it does not act as an extracellular mediator for cell death. It is still possible, however, that the production of other extracellular factors and the expression of their receptors may be involved.

At this point, we reasoned that if the expression of both a cytotoxic factor and its receptors is necessary for activation induced cell death, then cell density in culture should influence the extent of cell death induced by activation. When different densities (5 x 10^4 cells to 390 cells per 200 µl) of A1.1 cells were treated on an anti-CD3-coated plate for 18 h and assayed for cell viability by MTT, we found there was no significant difference in the ratio of the MTT readings of anti-CD3 treated over those of the control (Fig. V.2). Thus, there was no density effect observed. Since the range of cell density varied by more than 100 fold, a "death factor"/ receptor interaction should have been influenced by a change in density unless both factor and receptor are expressed in extreme excess and/or extremely low occupancy of the receptor is sufficient to induce cell death. That is, in most cases, we would expect that cell density in this range should affect cell death ratio. We have shown that at the density of 5 x 10^5 cells/ml there is 40 IU TNF/ml (Table V.3) Therefore, at 1.25 x 10^4 cells/ml (2,500 cells/200 µl well) there should be 1 IU TNF/ml, i.e., only half of the cells undergoing cell death. Based on the cell death pattern of L929 cells induced by TNF and the assumption that the production of extracellular factor and the expression of their receptors are responsible for the death of A1.1 cells, we predicted the cell death density effect on anti-CD3 induced cell viability change (Fig. V.3). This predicted pattern is dramatically different from that shown in Fig. V.2. Therefore, it is most likely that there is no role for cell interaction in activation induced cell death. However, it is still possible that factors produced by activated cells act in an autocrine fashion, or act intracellularly. In either case, we consider this an autonomous process.

In this section, we have demonstrated that activation induced

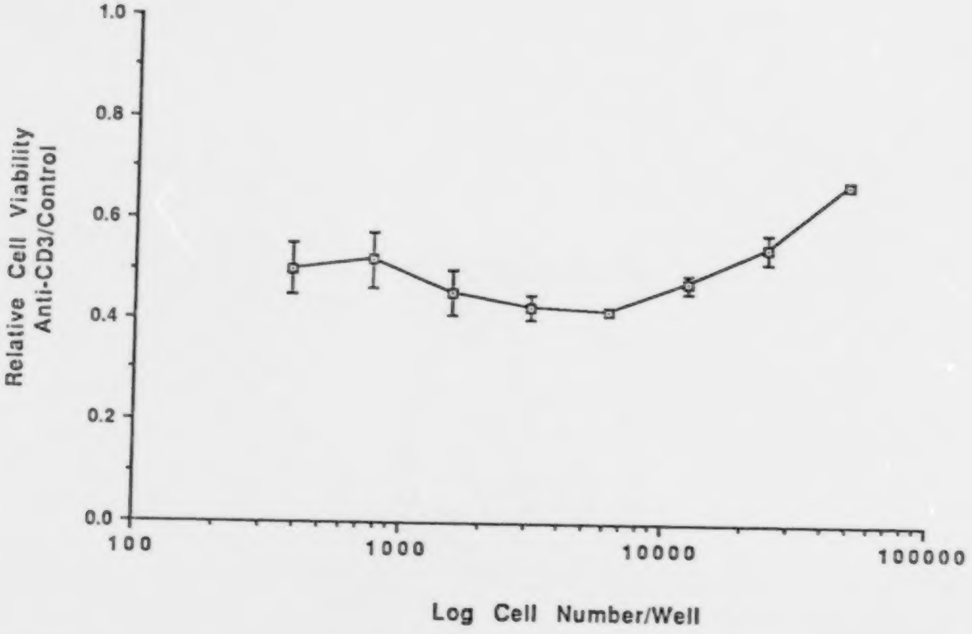


Fig. V.2. Cell density does not affect the efficiency of activation induced cell death in T cell hybridoma cells. A1.1 cells were titrated from 1 x 10^5 cells per well to 390 cells per well (the number of cells giving detectable MTT signal) in a 96-well tissue culture plate with/without anti-CD3 present. Cell viability was assayed by MTT 18 hr after stimulation. The relative cell viability is presented as the ratio of the anti-CD3 treated MTT reading over that of the control. Values below 1.0 indicate a loss of cell viability.

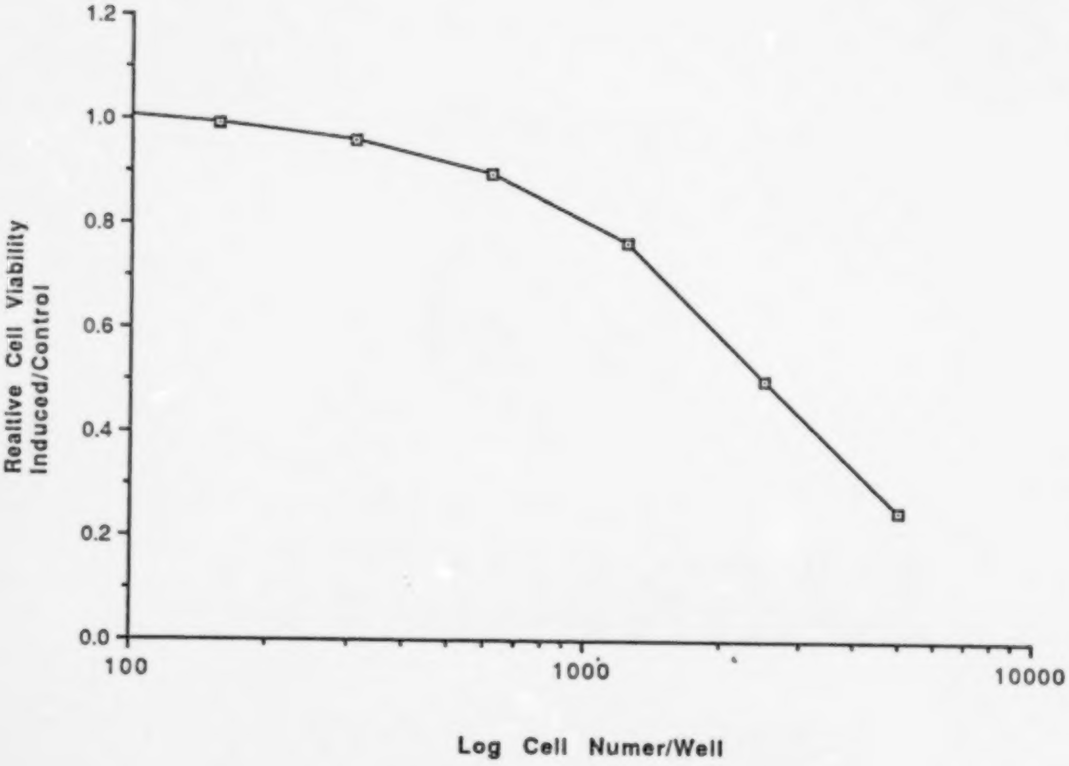


Fig. V.3. The predicted pattern of activation induced cell death in A1.1 cells mediated by "death factors". This pattern is based on the death of L929 cells induced by TNF-α. L929 cells were treated with different concentrations of TNF-α and the relative cell viability at 24 h was determined as the ratio of treated over control.

cell death in T cell hybridoma A1.1 cells is due neither to the production of newly synthesized extracellular factors, nor to the expression of receptors for these factors. It is, therefore, most likely that the mechanism of activation induced cell death is an intracellular autonomous process.

VI. THE ROLE OF ENDOGENOUS NUCLEASE IN ACTIVATION INDUCED CELL DEATH

Since DNA fragmentation is the hallmark of apoptosis, it has been proposed that the synthesis of endogenous endonucleases accounts for the requirement of protein and RNA synthesis by dying cells (Wyllie et al., 1984). However, the identification of such endonucleases have been somewhat controversial. Interestingly, Duke and Cohen (1984) have shown that an endonuclease with the capacity of generating DNA ladder is present in isolated nuclei from uninduced thymocytes, splenocytes and lymph node cells. They and Orrenius et al. (1989) suggest that nuclei contain a Ca^{++}, Mg^{++}-dependent endonuclease, inhibited by Zn^{++}, that is responsible for DNA fragmentation in apoptosis.

Here, we ask whether such endonuclease activity is present in the nuclei of A1.1 cells. We cultured these cells with ^{125}I-deoxyuridine overnight and isolated labeled nuclei from them with an ice cold Ca^{++} free buffer. Nucleus samples were incubated with and Ca^{++} and Mg^{++} in 37 °C water-bath. Every 5 minutes after incubation, EDTA containing nucleus lysis buffer was added to different samples to stop $Ca^{++}$and Mg^{++} dependent activities. The percentage of DNA in soluble form versus that in intact form was determined by centrifugation and assessment of gamma emission. As shown in Fig. VI.1, the percentage of soluble DNA in isolated nuclei increased as incubation proceeded. There were two sudden increases in the amount of soluble DNA, one at 20 minutes and the other at 45 minutes of incubation. As to be demonstrated later (Fig. VI.7), incubation of isolated nuclei with Ca^{++} at 37 °C produced the characteristic nucleosome-sized DNA ladder on an agarose gel. Therefore, the increase in soluble DNA was in fact due to the increase in DNA fragmentation. We thus conclude that the nuclei of A1.1 cells contain endonuclease activity.

It has been reported that a brief exposure of isolated normal thymocyte nuclei to micrococcal nuclease resulted in chromatin condensation and nucleosomal-sized DNA cleavage in a manner

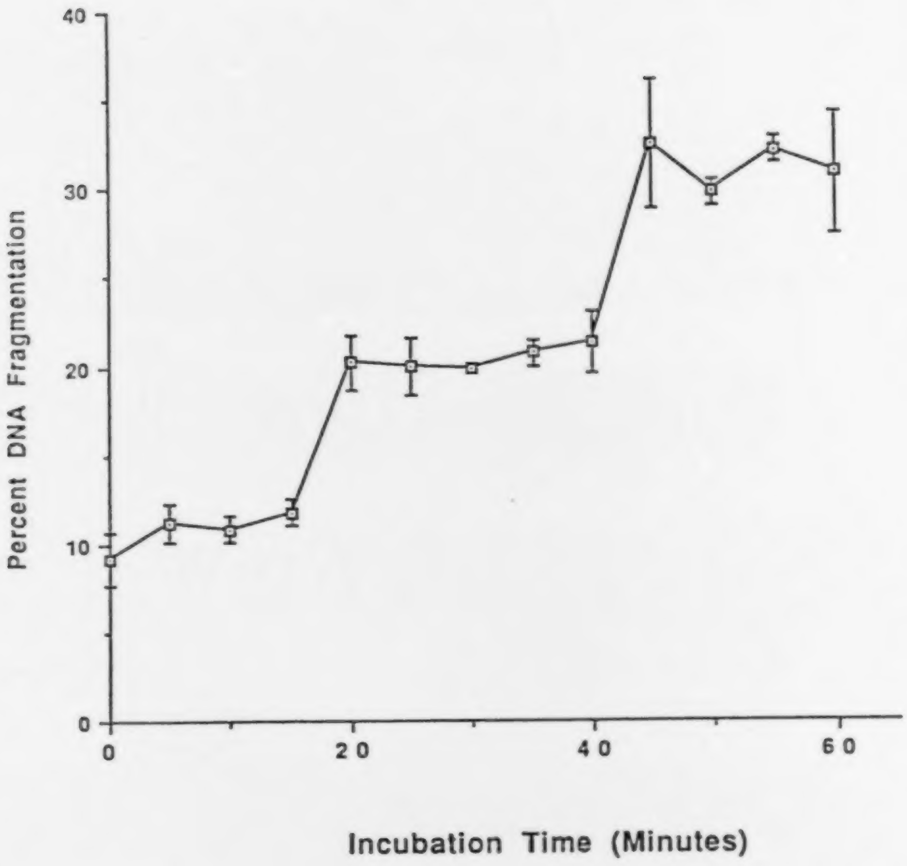


Fig. VI.1. Detection of the endogenous endonuclease activity present in nuclei. Uninduced A1.1 cells were labeled with ^{125}IUDR overnight. Nuclei were prepared and samples were incubated with 50 mM Mg^{++} and 50 mM Ca^{++} in a 37 °C water-bath. A buffer containing 200 mM EDTA was added to different nucleus samples every 5 minutes after incubation. DNA fragmentation in cell lysate was determined by measuring the amount of ^{125}I in the supernatant versus the total ^{125}I after centrifugation. This experiment was carried out only once due to the limitation of ^{125}I security. DNA fragmentation was also detected on agarose gel. Incubation of the nuclei with the same assay buffer at 4 °C for 2 h did not induce DNA fragmentation.

characteristic of cells undergoing apoptosis (Arends et al., 1990). It was concluded from such an experiment that the nuclear chromatin changes associated with apoptosis could be accounted for entirely by the activation of endonucleases. Thus, it would be of interest to determine the effect of inhibiting nuclease in activation induced cell death in our T cell hybridoma. Aurintricarboxylic acid (ATA) is known to interact with the nucleotide binding sites on nucleases and thus exert inhibitory effects on these enzymes (Hallich et al., 1977). It has been shown to inhibit apoptosis induced in thymocytes by glucocorticoid or calcium ionophore *in vitro* (McConkey et al., 1989). In our experiments, we tested the effect of ATA on activation induced cell death in T cell hybridomas. A1.1 cells were activated with anti-CD3 coated tissue culture plates with different concentration of ATA. Cell viability was assayed at 12 h after stimulation. As shown in Fig. VI.2, anti-CD3 alone induced significant loss in cell viability as we demonstrated before. Approximately 60-100 µM ATA completely inhibited activation induced cell death in the T cell hybridoma. Treatment of A1.1 cells with as high as 300 µM ATA did not interfere with cell proliferation as determined by 3H-thymidine incorporation (data not shown). To determine the effect of ATA on activation induced DNA fragmentation, we treated A1.1 cells with anti-CD3 with or without 75 µM of ATA, and isolated total genomic DNA at 24 and 48 h after stimulation. ATA completely blocked DNA fragmentation induced by anti-CD3 (Fig. VI.3). Furthermore, ATA also blocked DNA fragmentation produced by over growth in the control sample at 48 h. Thus, ATA is able to block apoptotic DNA fragmentation in the T cell hybridoma. However, when chemicals like ATA are used in biological systems, one must be cautious about drawing conclusions from their non-specific activities. For example, the ability of ATA to prevent cell death in activated T cell hybridomas might be due to their interference with cell metabolism. We therefore tested the effects of ATA on the synthesis of DNA, RNA and protein. A1.1 cells were cultured with 125 µM ATA and then pulsed with ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-leucine for 10 h with ATA present. The synthesis of DNA, RNA or protein were determined by scintillation counting. As shown in Fig. VI.4, ATA had no significant effects on all of them,

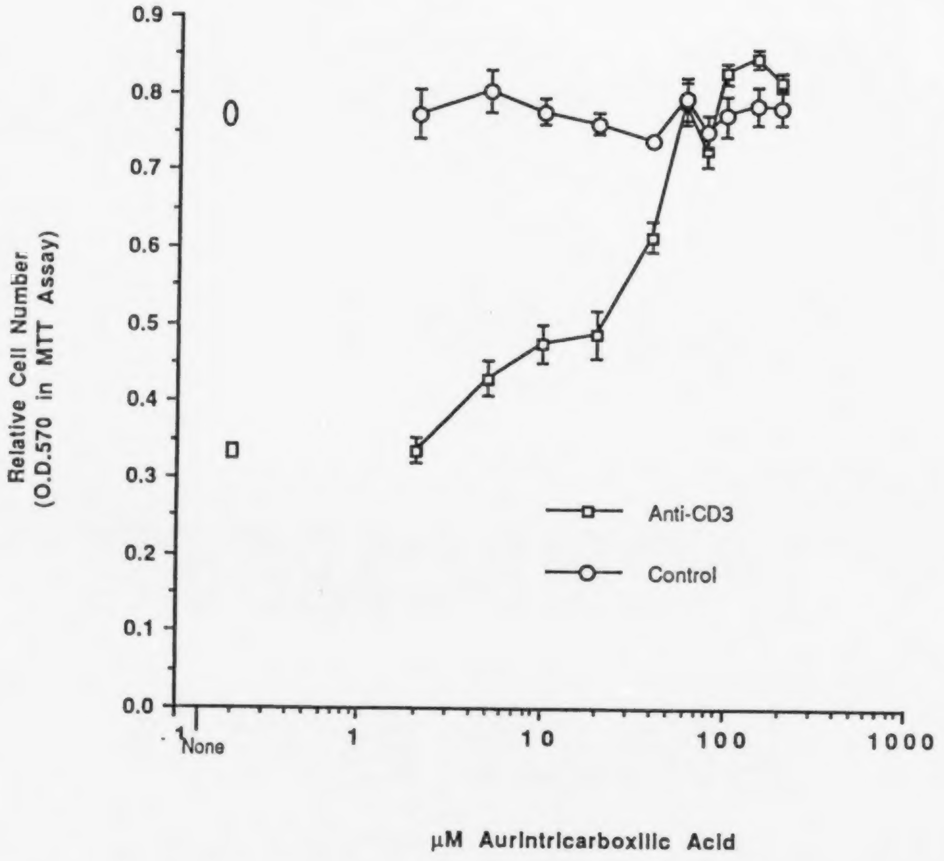


Fig. VI.2. Inhibition of activation induced cell death in a T cell hybridoma by ATA. A1.1 cells (1 X 10^5) were cultured in 200 μl of RPMI plus 5% FCS with various concentrations of ATA for 12 h. Cell viability was assayed by MTT.

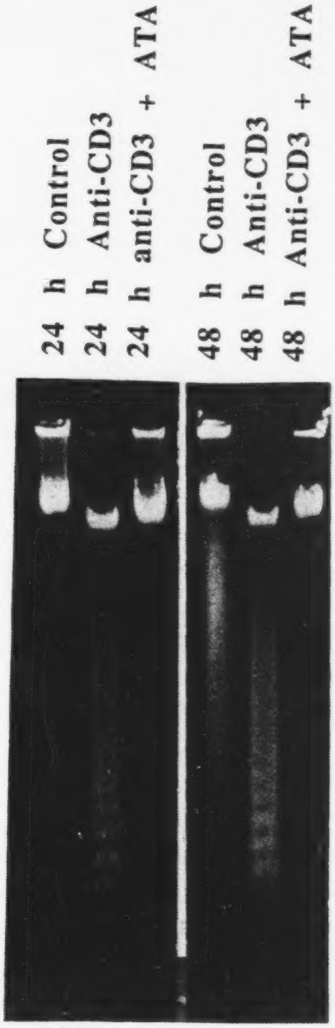

24 h Control
24 h Anti-CD3
24 h anti-CD3 + ATA
48 h Control
48 h Anti-CD3
48 h Anti-CD3 + ATA

Fig. VI.3. ATA inhibits anti-CD3 induced-DNA fragmentation in A1.1 cells. Cells were activated by anti-CD3 with/without 75 μM ATA present. DNA fragmentation was assayed by agarose gel electrophoresis. Control: untreated cells.

whereas actinomycin D and cycloheximide inhibited RNA and protein synthesis respectively. More significantly, we found that when activation induced cell death in the T cell hybridoma was completely inhibited by ATA, these cells still produced lymphokines to support the growth of CTL.L cells (Fig. VI.5). These results indicate that the inhibition of activation induced cell death of T cell hybridomas by ATA may have occurred through a direct effect on nucleases.

To investigate when ATA acts during the process of activation induced apoptosis relative to the kinetics of apoptotic events, we performed two parallel experiments with A1.1 cells labeled with ^{125}IUDR. These cells were activated on four anti-CD3 coated plastic 96-well plates. In one experiment, cells from two of the plates were assayed for DNA fragmentation and cell death respectively at different times after activation. DNA fragmentation was measured by counting the amount of ^{125}I in soluble form versus that in insoluble form after lysis and centrifugation, and cell viability was assayed by MTT. Fig. VI. 6A shows the percent of DNA fragmentation and percent of cell death at different times after activation. This clearly demonstrated that DNA fragmentation proceeds the loss of mitochondrial function (determined by MTT assay, as an indication of cell viability), i.e., significant DNA fragmentation could be detected at 4 h post stimulation, whereas significant loss of cell viability was only observed at 6-7 h after activation.

The second experiment was performed with the other two plates, to which we added ATA at different times after activation with anti-CD3, and similarly assayed DNA fragmentation at 9 h and cell viability at 11 h after anti-CD3 activation. Fig. VI.6B shows that addition of ATA up to 2 h post activation completely blocked both progression of cell death and DNA fragmentation. Addition at later times partially inhibited ultimate DNA fragmentation and cell death. The effects of adding ATA on ultimate DNA degradation at different times post-activation showed strong correlation with the

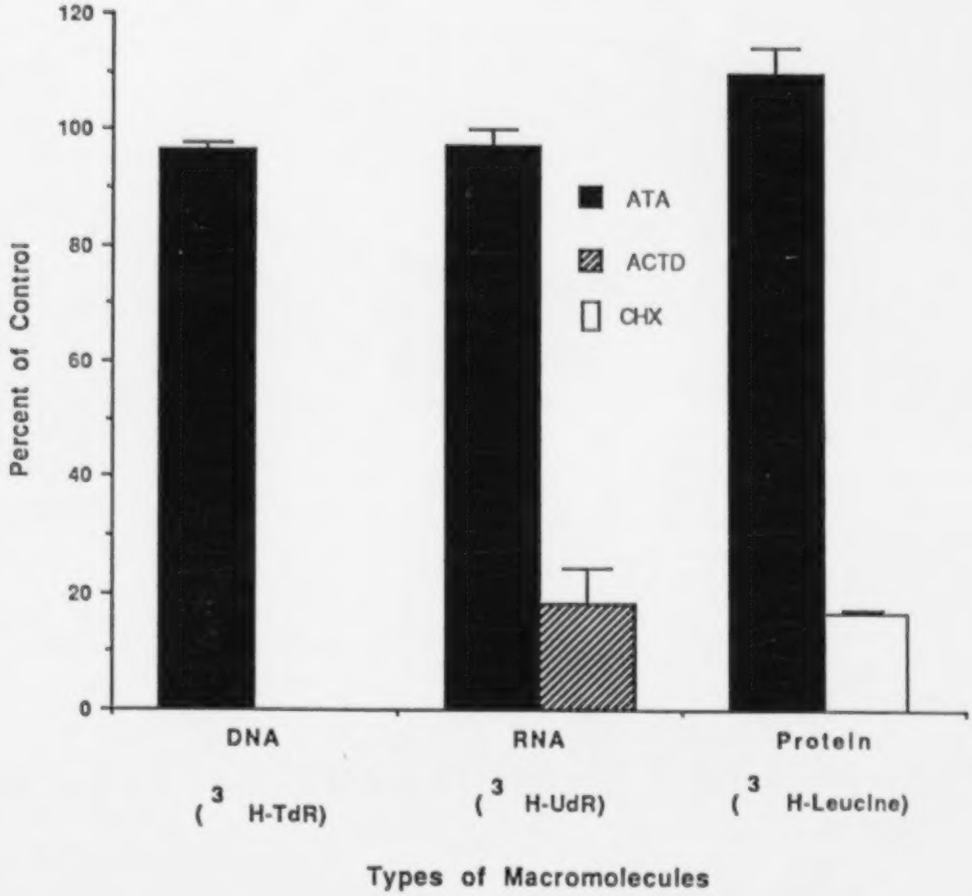


Fig. VI.4. The inability of ATA to inhibit DNA, RNA and protein synthesis. Unstimulated A1.1 cells (5 x 10^4) were cultured with 125 μM of ATA for 2 h and then pulsed with ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-leucine for 10 h. The amounts of macromolecule synthesis were determined by scintillation counting. Actinomycin D and cycloheximide were used at 2.5 μM and 1.5 μM respectively.

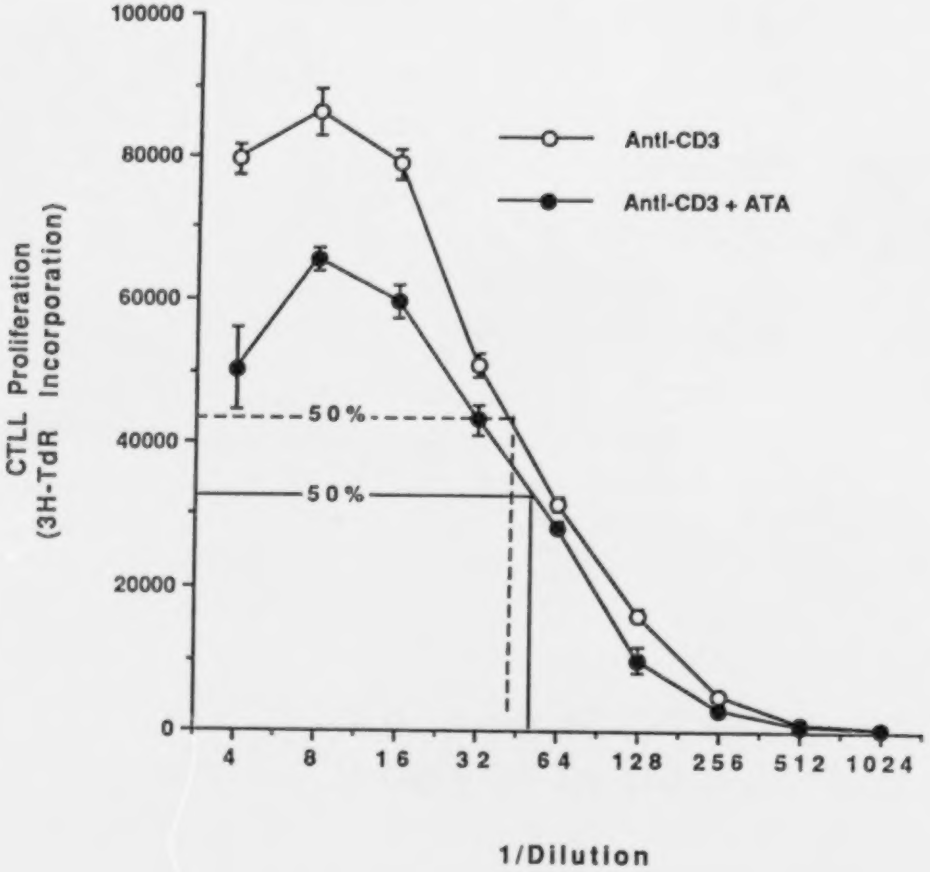
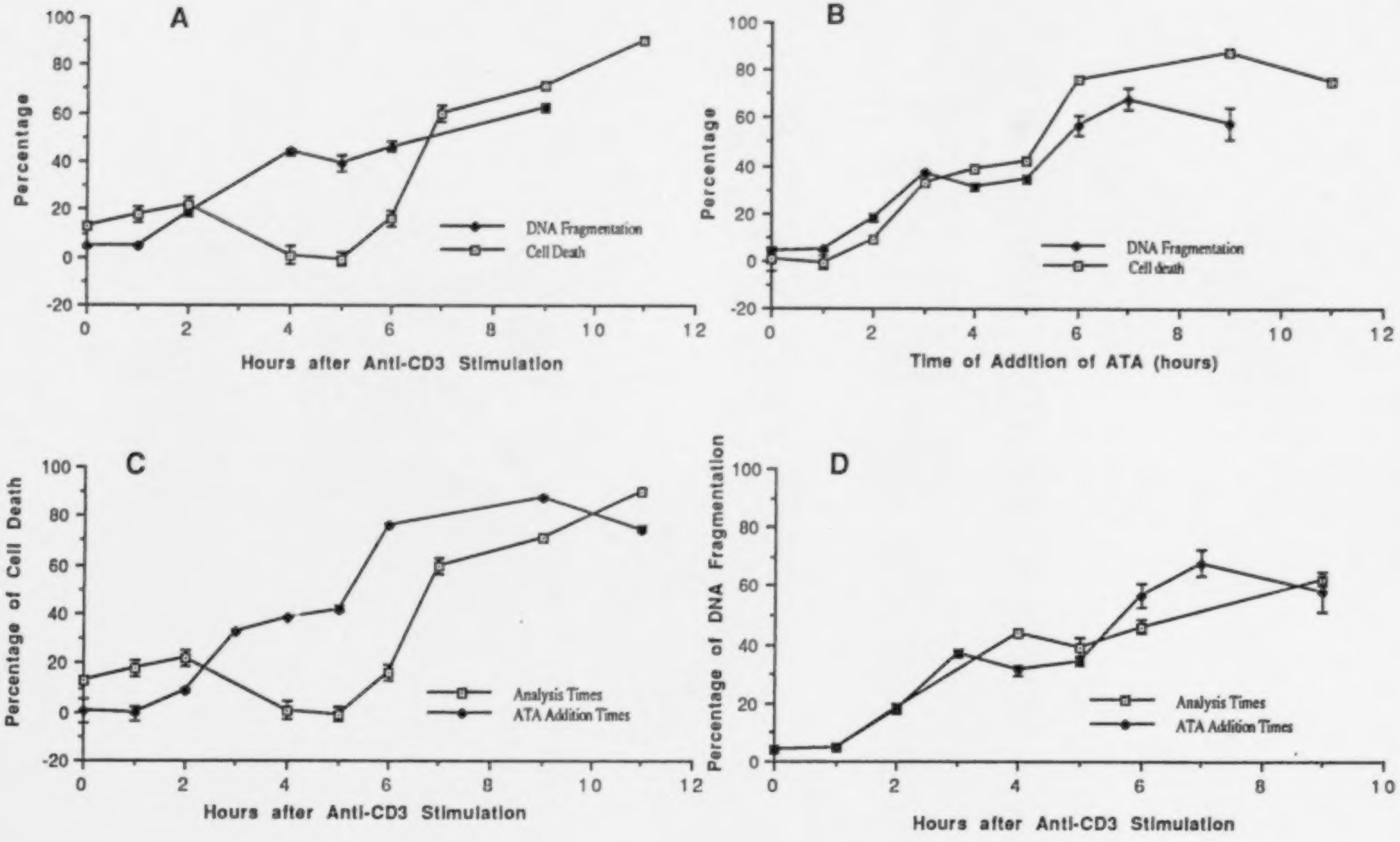


Fig. VI.5. The effect of ATA on lymphokine production by anti-CD3 activated A1.1 cells. A1.1 cells were activated with anti-CD3 coated plastic for 8 h with/without 75 μM ATA. The supernatant was collected and diluted with RPMI media supplemented with 5% FCS. Lymphokine production was determined as the ability to support CTL.L cell growth (expressed by ^{3}H-TdR incorperation).

Fig. VI.6. The role of nuclease in activation induced cell death in T cell hybridoma. ^{125}IUDR labeled A1.1 cells were activated on four anti-CD3 coated plates. At various times (shown above) after activation, cells in two of the plates were assayed for percentage of cell death by MTT, and for percent of DNA fragmentation by counting the radioactivity in solution and pellet after lysis and centrifugation (A); At the times stated after activation 75 μM ATA were added to the other two plates and percent of cell death and DNA fragmentation were assayed at 11 h after anti-CD3 treatment. This experiment was repeated with similar results. Percent of cell death of different time points after activation versus different ATA addtition time after activation is presented in (C). Percent of DNA fragmentation of different time points after activation versus different ATA addtition time after activation is presneted in (D).


A
Percentage
100
80
60
40
20
0
-20
0
2
4
6
8
10
12
Hours after Anti-CD3 Stimulation
DNA Fragmentation
Cell Death
B
Percentage
100
80
60
40
20
0
-20
0
2
4
6
8
10
12
Time of Addition of ATA (hours)
DNA Fragmentation
Cell death
C
Percentage of Cell Death
100
80
60
40
20
0
-20
0
2
4
6
8
10
12
Hours after Anti-CD3 Stimulation
Analysis Times
ATA Addition Times
D
Percentage of DNA Fragmentation
100
80
60
40
20
0
0
2
4
6
8
10
Hours after Anti-CD3 Stimulation
Analysis Times
ATA Addition Times

extent of DNA fragmentation at those times (Fig. VI.6D), indicating that ATA effectively freezes DNA fragmentation at the time of addition and no further degradation occurs. The effects of adding ATA at different times on ultimate DNA fragmentation and ultimate cell death also showed correlation(Fig. VI.6B). Further, inhibition of ultimate cell death by adding ATA at different times correlates with DNA fragmentation at those times (Fig. VI.6A and B). Thus, addition of ATA at or near the time of DNA fragmentation prevented cells from entering the death pathway. However, no significant correlation was observed between the effects of adding ATA on ultimate cell death at different times versus cell death at those times (Fig. 6.C). This again suggests DNA fragmentation precedes cell death and ATA could not prevent the death of cells which already have fragmented their DNA.

DNA fragmentation has always been considered a characteristic of cells which have died by apoptosis as distinct from those which died by necrosis. What, however, is the relationship between DNA fragmentation and cell death? The ability of the nuclease inhibitor ATA to inhibit both cell death and DNA fragmentation implies that DNA fragmentation is a key event in activation induced apoptosis. Such a conclusion agrees with McConkey et al. (1989), who reported that ATA could also interfere with glucocorticoid and A23187 induced cell death in thymocytes. To determine whether ATA can directly block the function of endonuclease thought to be involved in apoptotic cell death, we isolated nuclei from T cell hybridoma A1.1 cells and exposed them to Ca++ and Mg++ to induce characteristic DNA fragmentation as described (Duke and Cohen, 1984). Addition of 100 μM ATA effectively blocked this DNA fragmentation (Fig.VI.7), and it is in this dose range that ATA blocks activation induced cell death in this T cell hybridoma (Fig. VI.2). Together with the data in Fig. VI.6, these results suggest that it is by inhibition of DNA fragmentation that ATA prevents activation induced apoptosis. Further, this suggests that DNA fragmentation is required for this form of

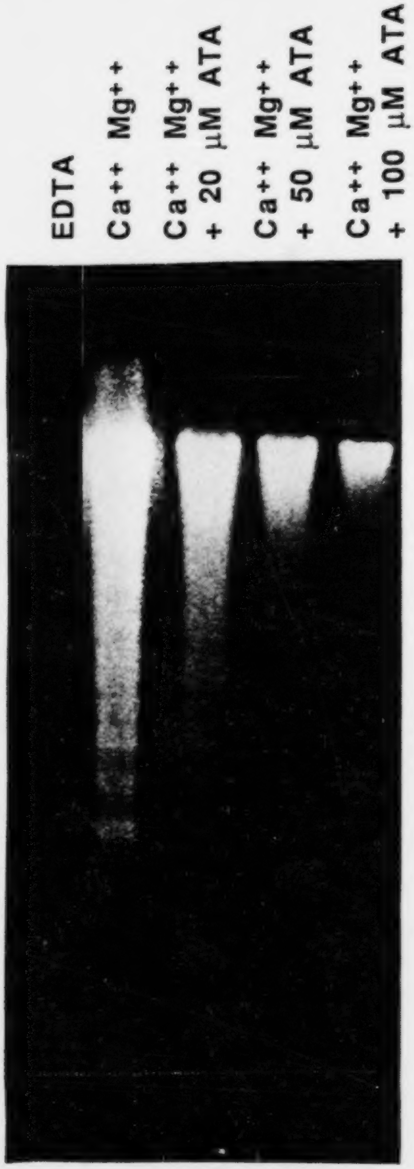

EDTA
Ca^{++} Mg^{++}
Ca^{++} Mg^{++} + 20 µM ATA
Ca^{++} Mg^{++} + 50 µM ATA
Ca^{++} Mg^{++} + 100 µM ATA

Fig. VI.7. Activity of the endogenous endonuclease in isolated nuclei. Nuclei were isolated from uninduced A1.1 cells and incubated at 37 °C for 90 minutes in assay buffer with/without EDTA. Various concentrations of ATA were added just before incubation. Fragmented DNA was extracted from the nucleus samples and analyzed on agarose gel.

apoptosis.

Above, we have considered that DNA fragmentation is the first irreversible step in activation induced apoptosis, before which cells have the potential to survive. This assumption could explain why ATA can prevent cell death even when added 2-4 h after activation. In Chapter IV, we described an experiment in which A1.1 cells were stimulated with phorbol ester plus ionomycin or with anti-CD3 for different times and examined for cell death at 14 h after the initial stimulation. As shown in Fig. IV.8, stimulation for less than 3 h failed to induced cell death, while 3-4 h of activation was sufficient to induce cell death. Since the commitment to death corresponds to the onset of DNA fragmentation, it is likely that the irreversible DNA fragmentation is responsible for this commitment.

We have shown in chapter III that injection of antibodies to the TCR-CD3 complex induces characteristic apoptosis in immature thymocytes. To examine the effect of ATA on this *in vivo* activation induced cell death, we injected mice intravenously with anti-CD3 antibodies, and intraperitoneally with ATA. Thymuses were removed 24 h later, weighed, and the total thymocytes counted. The loss in the number of thymocytes induced by anti-CD3 antibodies was blocked by 1 mg ATA (Fig. VI.8). Further, the DNA fragmentation in immature thymocytes induced by anti-CD3 was blocked by ATA (Fig. VI.9).

Overall, we have demonstrated that nuclease inhibitor ATA interferes with activation induced cell death at or near the step of DNA fragmentation and that directly blocks endogenous endonuclease function in isolated nuclei. *In vivo* administration of ATA prevent anti-CD3 induced apoptosis in immature thymocytes. These experiments support the essential role for DNA fragmentation in activation-induced apoptosis.

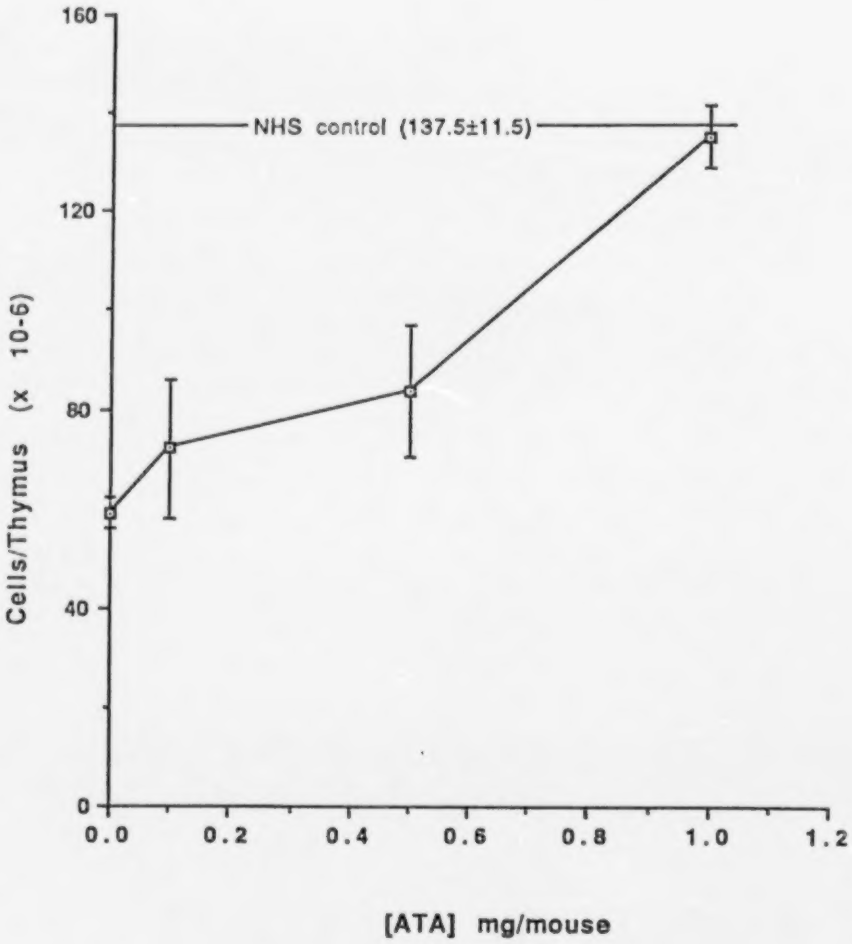


Fig. VI.8. The effect of ATA on activation induced cell loss in thymocytes *in vivo*. Six-week-old Balb/c mice were injected i.p. with 0.2 ml concentrated 145-2c11 supernatant with/without different amounts of ATA. Thymuses were removed 24 h later and the number of thymocytes were counted. A separate experiment showed that the treatment of control mice with different ATA doses (0.1 to 1 mg/mouse) did not result in significant change in thymocyte count.

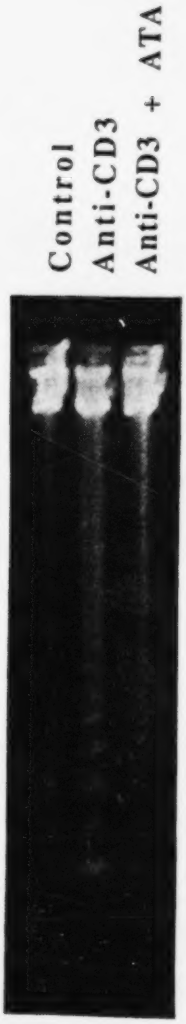

Control
Anti-CD3
Anti-CD3 + ATA

Fig. VI.9. Inhibition of cell death in the thymus induced by anti-CD3 *in vivo*. Six week-old Balb/c mice were injected with anti-CD3 antibody at 0 h. Five hundred µg ATA were injected at -4 h, 2 h and 6 h. DNA fragmentation was assayed at 10 h on an agarose gel.

VII. SIGNAL TRANSDUCTION AND ACTIVATION INDUCED CELL DEATH

Antigen recognition and response of T lymphocytes are mediated by antigen-receptors. The capacity of the T cell receptors to transduce the ligation signals to multiple biological cascades is the essential event of T cell activation. The T cell antigen receptor comprises a clonotypic $\alpha\beta$ heterodimer providing antigen specificity, associated noncovalently with CD3 which includes multiple invariant dimers (Manolios et al., 1991). The generation of second messengers after occupancy or cross-linking of the antigen receptor-CD3 complex also requires accessory molecules on the cell surface. It is interesting that both mature and immature T cells bear the same T cell receptor-CD3 complex, however, it mediates positive, negative and clonal selection as a result of recognition of antigen and MHC. It is very possible that the differential responsiveness of T lymphocytes at different stages might reflect some maturation related differences in signal transduction. Therefore, the elucidation of signal transduction involved in activation-induced cell death is of great interest in understanding T cell maturation.

Many studies have been devoted to the signal transduction pathways of T cell activation, however, it is still not known exactly how the ligation of the T cell receptor leads to the biological responses. A speculative model for the proximal events has been proposed. The engagement of TCR with antigen plus MHC activates tyrosine kinases associated with the T cell receptor and accessory molecules. These activated tyrosine kinases then phosphorylate a number of intracellular substrates, including phospholipase C. Phosphorylation of phospholipase C results in hydrolysis of membrane phosphotidylinositols, leading to the transient increase in diacylglycerol (DAG) and inositol phosphates (Klausner and Samelson, 1991). DAGs are the physiological activator of PKC and inosital phosphates alter the cytoplasmic Ca^{++} concentration. Both Ca^{++} ions and protein kinase C have been implicated in the early events leading to T cell activation (June et al., 1990). Through some unknown mechanism (sometimes referred to as the "black box"), these messengers then activate genes in the nucleus, causing activation of responding cells. Using T cell hybridoma A1.1 as previously described, we have shown that a number of activation stimuli, including antigen presented by appropriate antigen presenting cells, Con A, plastic coated with anti-CD3 or anti-TCRβ, calcium ionophore A23187, and phorbol ester plus ionomycin, all led to rapid DNA fragmentation and cell death (Fig IV.1).

One way to activate T cells, causing them to proliferate and to produce lymphokines, is to supply a combination of calcium ionophore and phorbol ester to mimic signals provided by stimulation through T cell receptors, implying both calcium influx to cytosol and translocation of protein kinase C from cytosol to the cell membrane are required for T cell activation (Gelfand et al., 1987). It has been demonstrated that calcium influx is also required for glucocorticoid induced cell death in thymocytes (McConkey et al., 1990). We performed studies, therefore, to investigate the role of calcium in the induction of DNA fragmentation by anti-CD3 in our T cell hybridoma. We activated A1.1 cells with anti-CD3 antibody-coated plastic in calcium free medium supplemented with different concentrations of calcium and assayed DNA fragmentation 12 h after stimulation. We found that the extent of DNA fragmentation was dependent on calcium concentration in the medium (Fig. VII.1). To confirm this observation, we chelated calcium in RPMI 1640 using different concentrations of EGTA. Cell viability was determined by flourescein isothiocyanate in the presence of 5% fetal calf serum at 10 h post stimulation. The induction of cell death of A1.1 cells was reduced in a dose dependent manner following stimulation with anti-CD3 (Fig. VII.2). These experiments clearly demonstrated that Extracellular Ca^{++} is essential for activation-induced cell death in the T cell hybridoma.

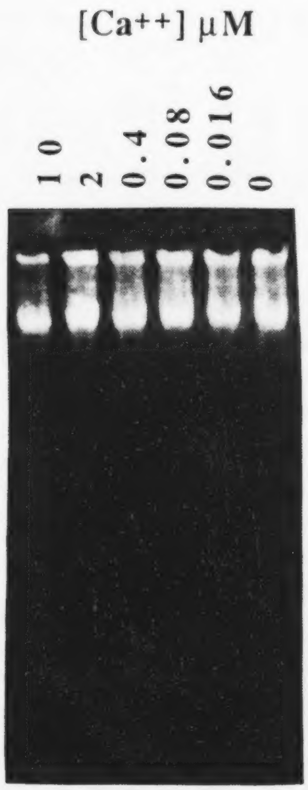


Fig. VII.1. The requirement for Ca^{++} by A1.1 cells for the induction of DNA fragmentation. A1.1 cells were activated with anti-CD3 coated plastic in Joklik modified MEM medium supplemented with different concentrations of Ca^{++}. Total DNA was analysed12 h after stimulation.

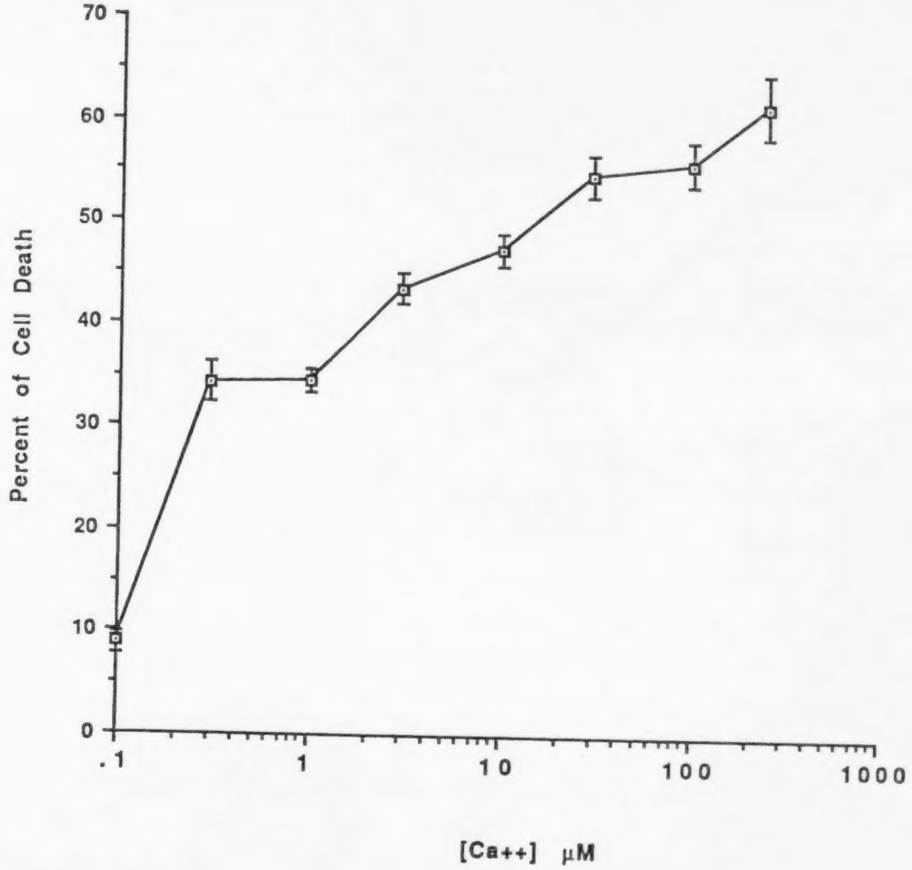


Fig. VII.2. The requirement for Ca^{++} by A1.1 cells for activation induced cell death. A1.1 cells were activated with anti-CD3 coated plastic in RMPI supplemented with 5% FCS. Various concentrations of EGTA were added to reduce free Ca^{++} in the medium according to Portzehl et al., (1964). Ten h after stimulation, cell viability (percent of cell death) was assayed by the FITC uptaking.

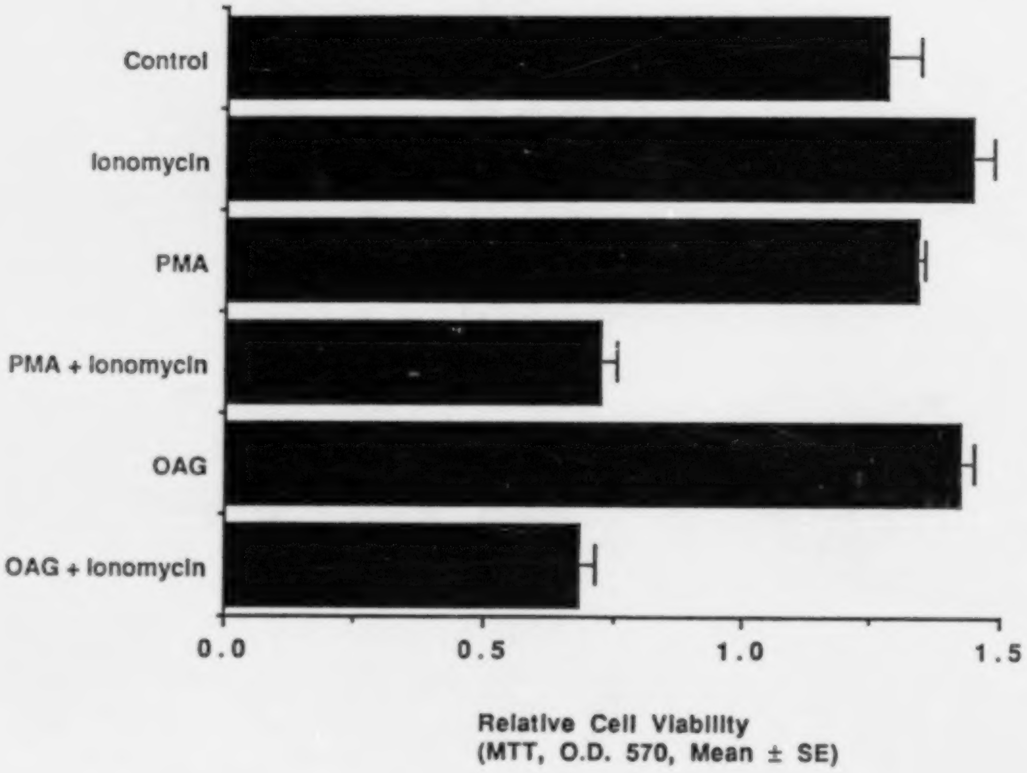


Fig. VII.3. Induction of cell death in A1.1 cells by activation of PKC and Ca^{++} influx. A1.1 cells were treated with 25 nM ionomycin with/without 62.5 nM of OAG or 10 nm PMA. Cell viability was assayed by MTT at 12 h after stimulation.

In T lymphocytes phorbol esters, potent activators of PKC, act as costimulators with perturbation of the T cell receptors to increase cell proliferation and production of T cell growth factor IL-2 and the expression of IL-2 receptors (Isakov et al., 1987). To test the function of PKC in activation-induced cell death we included either PMA or OAG, another diacylglycerol analogue, to stimulate A1.1 cells in combination with ionomycin. Twelve hours after stimulation cell viability was determined by MTT. As shown in Fig. VII.3, either ionomycin or diacylglycerol analogues did not change cell viability, while the combination of ionomycin with either PMA or OAG induced significant reduction in cell viability. These results indicate that activation of PKC in the presence of calcium ionophore represents at least one way to activate the cell death pathway in the T cell hybridoma.

In contrast to the above evidence indicating that PKC is involved in activation induced cell death, it has been reported that DNA fragmentation was observed in thymocytes treated with concanavalin A plus the PKC inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) (McConkey, et al., 1989), showing that thymocytes become apoptotic only when PKC is inactive. This confliction stimulated us to evaluate the effects of inhibition of PKC on activation induced cell death in our T cell hybridoma system. As shown in Fig. VII.4, addition of H7 or staurosporin, another reported PKC inhibitor (O'Flaherty and Jacobson, 1989), to anti-CD3-activated A1.1 cells caused a dose dependent reduction in activation-induced cell death. At 3 $X10^{-4}$ M H7 and 6 X 10^{-8} M staurosporin death of A1.1 induced by anti-CD3 was completely prevented. When the effect of staurosporin was tested on DNA fragmentation of A1.1 cells 8 hour after activation, it was found to completely block DNA fragmentation induced by anti-CD3 (Fig. VII.5). However, experiments with PKC inhibitors such as staurosporin do not give definite answers about the role of PKC because these inhibitors also inhibit other kinases such as tyrosine

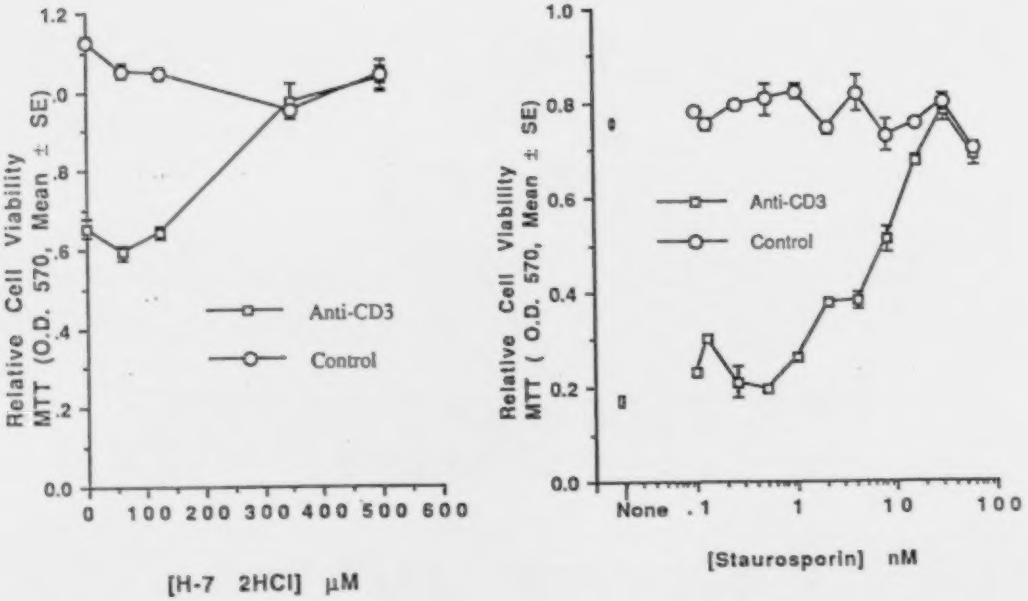


Fig. VII.4. Inhibition of activation-induced cell death in the T cell hybridoma , A1.1, by H-7 or staurosporin. A1.1 cells were activated on anti-CD3-coated plastic with various concentrations of H-7 or staurosporin present. Cell viability was assayed by MTT at 24 h after stimulation.

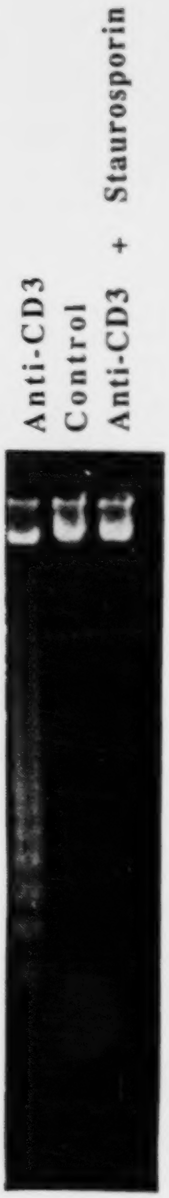

Anti-CD3
Control
Anti-CD3 + Staurosporin

Fig. VII.5. Inhibition of activation induced DNA fragmentation in a T cell hybridoma. A1.1 cells were activated on anti-CD3 coated plastic with/without 60 nM staurosporin. DNA fragmentation was assayed at 8 h post-activation.

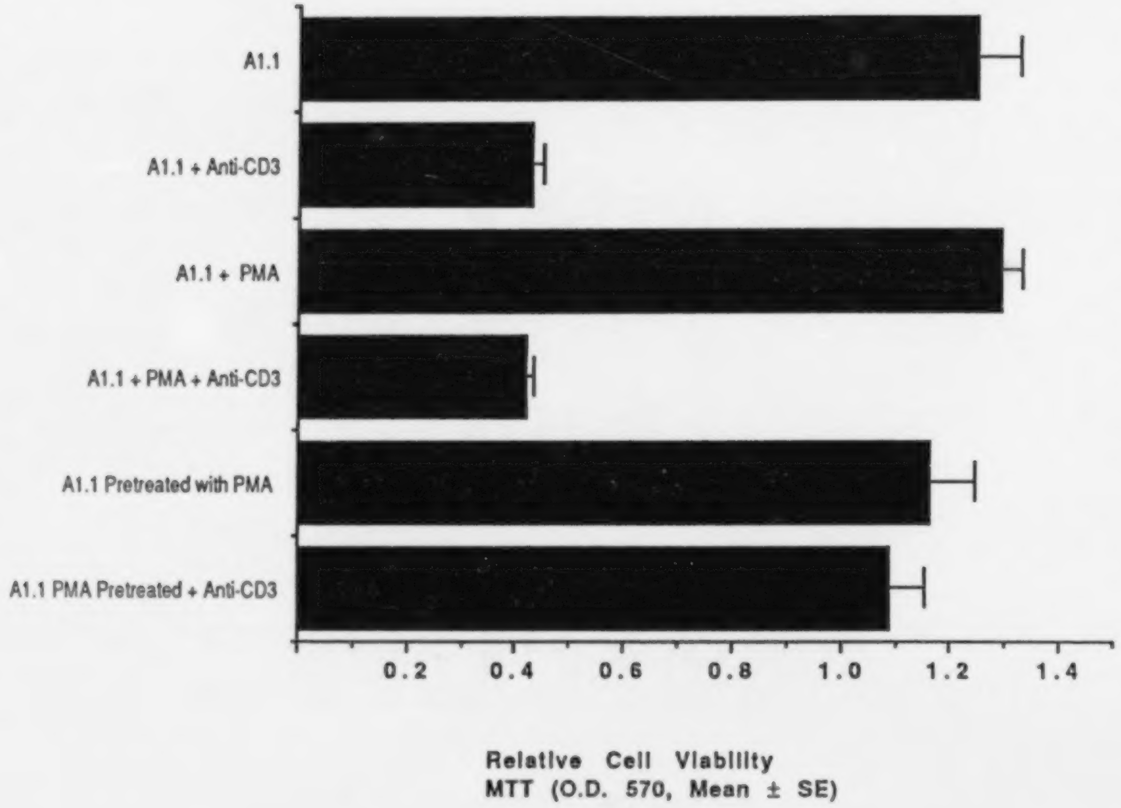


Fig. VII.6. Depletion of PKC by pretreatment with PMA prevents activation induced cell death in T cell hybridoma. A1.1 cells were pretreated with 2 µM PMA for 12 h and then treated with anti-CD3 coated plastic for 12 h. Alternatively, 0.01 µM PMA was added to cells at the time of activation by anti-CD3. Cell viability was assayed by MTT.

kinases, and tyrosine kinases are very likely to phosphorylate substrates other than PLC.

A more specific approach to inactivate PKC is by depletion. It has been demonstrated that in many cell types, including lymphocytes, overnight incubation with a high concentration of phorbol esters could remove PKC by increasing the activity of a degradation enzyme (Woodgett and Hunter, 1987). We incubated A1.1 cells with 2 µM of PMA for 12 h and then treated with anti-CD3. As demonstrated before, nontreated cells died after activation, which was not prevented by addition of PMA. Nevertheless, PMA-pretreated cells were resistant to activation-induced cell death (Fig. VII.6). Due to the specificity of PMA, this might be our strongest evidence to suggest a role for PKC in activation-induced death. These results are consistent with Ojeda et al. (1989), who found that apoptosis of mouse thymocytes induced by hydrocortisone *in vitro* could be prevented by H-7. McConkey et al. (1990) observed that PMA or IL-1 could inhibit apoptosis in thymocytes induced with A23187 or anti-CD3. A possible explanation is that PKC may play different roles in activation induced cell death in different investigation systems

We have demonstrated that activation of PKC and Ca^{++} influx could be one set of signals for cell death of A1.1. These signals may also be involved in activation through the T cell receptors. However, the activation of other kinases upon T cell receptor ligation may also be able to provide signals for cell death because the PKC inhibitors could also inhibit tyrosine kinases. Therefore, a definite conclusion about which kinase is involved in activation induced cell death remains to be investigated.

VIII. CYCLOSPORIN A AND TRANSFORMING GROWTH FACTORS INHIBIT ACTIVATION INDUCED CELL DEATH

A. Cyclosporin A Blocks Activation Induced Cell Death *In Vitro* and *In Vivo*

Cyclosporin A, a cyclic undecapeptide (11 amino acid peptide) obtained from the fungi imperfecti, *Cylindrocarpon lucidum* and *Tolypocladium inflatum*, is a potent immunosuppressive agent (Borel et al., 1976). It suppresses the immune system mainly through its effect on T lymphocytes, and has fewer side effects associated with it than other conventional immunosuppressive agents. It has, therefore, been widely used in organ transplantation and autoimmunity treatments. However, it was found that some patients and animals developed post-cyclosporin A autoimmunity after its administration (Glazier et al., 1983). In experiments with mice, this autoimmune phenomenon was transferable with T cells (Cheney and Sprent, 1983; Sakaguchi and Sakaguchi, 1988). Because the removal of the thymus prevented this kind of autoimmunity, it was concluded that the thymus is critical for the development of the autoreactive T cells (Sorokin et al., 1986). Several mechanisms of the post-cyclosporin A autoimmune phenomenon have been postulated, including that of T repertoire alteration secondary to reduced expression of MHC on thymic epithelia (Cheney and Sprent, 1983), an effect on the development of regulatory cells (Sorokin et al., 1983; Sakaguchi and Sakaguchi, 1988) or interference with the process of negative selection which eliminates autoreactive T cells (Jenkins, et al., 1988). The following experiments were designed to test the effects of cyclosporin A on activation-induced apoptosis using our established *in vivo* and *in vitro* systems, and to provide us with information about the relationship between activation-induced cell death and negative selection.

It is known that cyclosporin A inhibits T cell activation by preventing IL-2 mRNA accumulation and cell proliferation (Elliot et al., 1984). To determine what effect, if any, cyclosporin A has on activation-induced cell death *in vitro*, we applied cyclosporin A in our T cell hybridoma system. A1.1 cells were stimulated with anti-CD3-coated plastic tissue culture plates in the presence of various concentrations of cyclosporin A. The percent of viable cells was determined by fluorescein isothiocyanate in the presence of 5% fetal calf serum at 8.5 h post stimulation. As shown in Fig. VIII.1, cyclosporin A inhibited anti-CD3-induced cell death in a dose-dependent manner, with complete inhibition occurring at 120 ng/ml. This dose of cyclosporin A corresponded to the dose required for the inhibition of lymphokine production by activated lymphocytes *in vitro* by Shevach (1985). We then activated A1.1 cells with specific peptide antigen plus antigen presenting cells, or concanavalin A, or anti-CD3 coated plastic, with or without cyclosporin A (200 ng /ml). Relative cell viability was assayed by MTT. As shown in Fig. VIII.2, A1.1 cells died and lost their ability to convert MTT after stimulation with various reagents, which was completely prevented by cyclosporin. By light microscopy, cyclosporin A inhibited formation of membrane blebs and apoptotic bodies associated with activation induced cell death in the T cell hybridoma (Fig. VIII.3).

As previously demonstrated, the genomic DNA of apoptotic A1.1 cells was fragmented into a nucleosome-sized ladder. Here, we examined the effect of cyclosporin A on such activation-induced DNA fragmentation in T hybridoma cells. Fig. VIII.4 shows that anti-CD3-induced characteristic DNA fragmentation as demonstrated earlier. The application of cyclosporin A prevented DNA fragmentation in anti-CD3 activated A1.1 cells. Thus, cyclosporin A prevents loss of cell viability, morphological changes and genomic DNA fragmentation in the T cell hybridoma A1.1 following activation via the T cell receptor complex. This might represent a general effect of cyclosporin A on activation induced cell death.

One mechanism by which the immune system discriminates self from non-self is the deletion of autoreactive T cells during their development in the thymus. The drug cyclosporin A has been

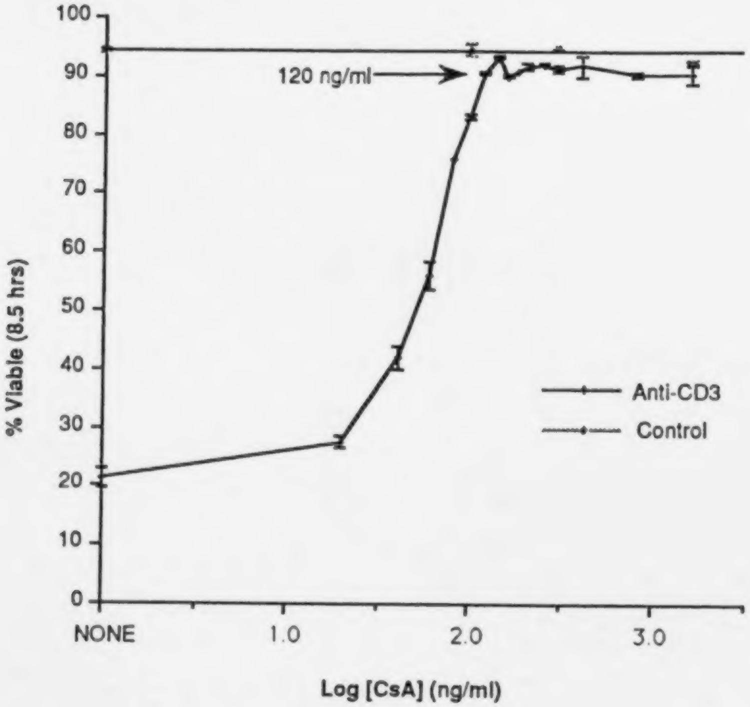


Fig. VIII.1. Effect of cyclosporin A on activation-induced cell death in the T cell hybridoma A1.1. A1.1 cells (1 X 10^5) were activated with anti-CD3-coated plastic in the presence of different concentrations of cyclosporin A (CsA). Mean percentage of viable cells ± SE of three replicates was determined 12 h after stimulation by the fluorescein isothiocyanate exclusion method.

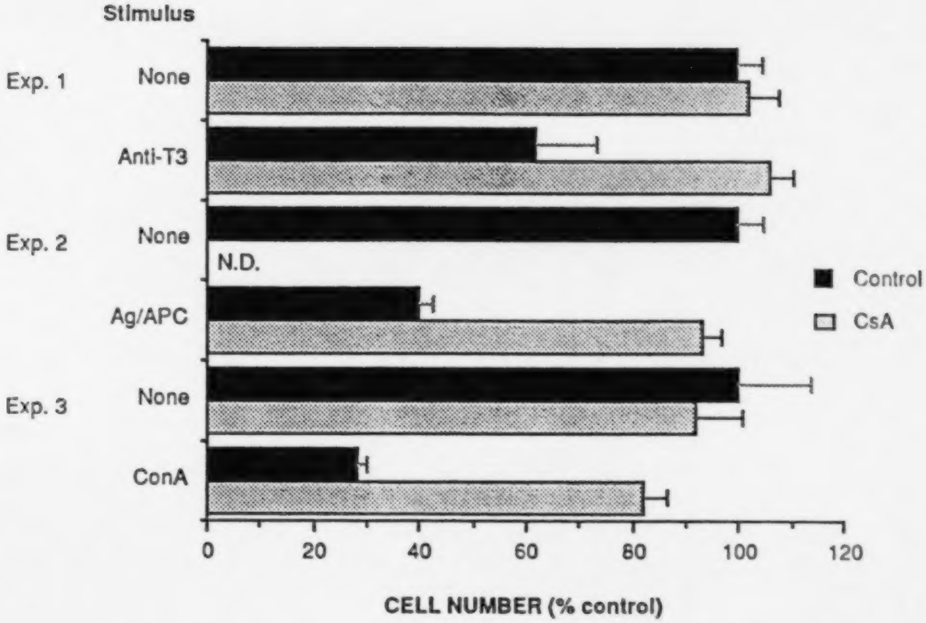


Fig. VIII.2. Cyclosporin A blocks cell death in T cell hybridoma A1.1 induced by different stimuli. A1.1 cells were activated with anti-CD3 coated plastic, poly-18 plus APC (TA3), or concanavalin A, with or without (control) cyclosporin A (200 ng/ml). Relative cell viability was determined by MTT staining at 24 h after activation and expressed as percentage of control.

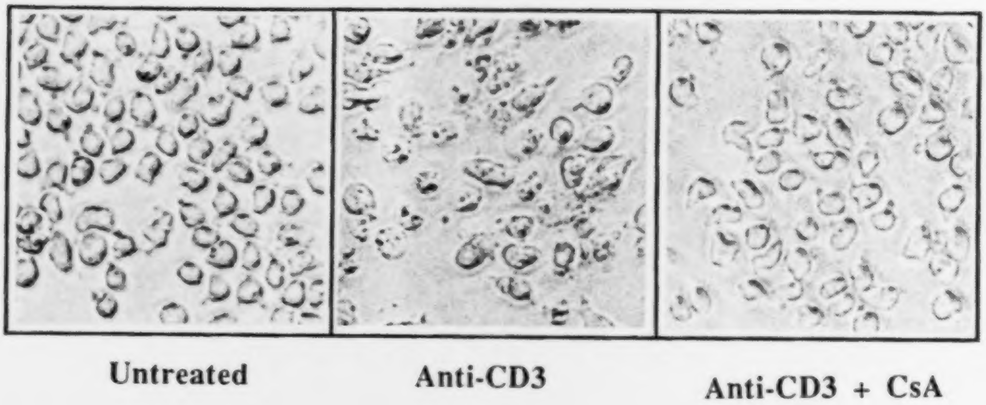


Fig. VIII.3. Cyclosporin A prevents activation-induced morphological changes in A1.1 cells. A1.1 cells were cultured in a 96-well plastic tissue culture plate coated with anti-CD3 with or without cyclosporin A (CsA, 200 ng/ml). Cells were viewed with an inverted microscope, and photograpges were taken12 h after activation.

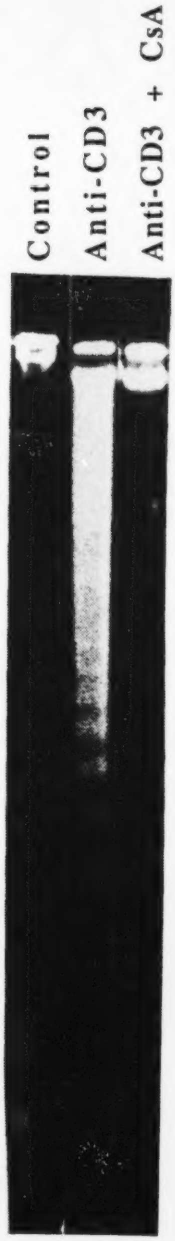


Fig. VIII.4. Cyclosporin A inhibits activation induced DNA fragmentation in A1.1 cells. A1.1 cells were activated in anti-CD3 coated 75 cm^2 tissue culture flasks. Cyclosporin A was added at 250 ng/ml. DNA was extracted at 12 h post stimulation. Nontreated cells were used as control.

shown to interfere with this process and to allow the escape of the potentially autoreactive T cells to the peripheral mature T cell might repertoire (Gao et al., 1988; Jenkines et al., 1988), which be related to the development of autoimmunity (Cheney and Sprent, 1985; Sakaguchi and Sakaguchi, 1988). It has been clearly demonstrated by our experiments and by others' (Smith et al., 1989; Murphy et al., 1990) that activation of immature thymocytes leads to cell death, and this has been proposed as a mechanism of negative selection. To determine whether cyclosporin A would inhibit activation induced cell death in immature thymocytes *in vivo*, mice were treated with anti-CD3 or anti-CD3 plus cyclosporin A. The dose chosen was that shown to interfere with clonal deletion in the thymus (Gao et al., 1988). Fig. VIII.5 shows cyclosporin A clearly inhibits DNA fragmentation in thymocytes induced by anti-CD3 *in vivo*. This result suggests that one mechanism by which cyclosporin A affects T cell development is by directly interfering with the intrathymic deletional process and allowing the escape of autoreactive T cells. It also suggests a link between activation-induced cell death and negative selection.

B. Transforming Growth Factors Inhibit Activation-Induced Cell Death

Transforming growth factors (TGF) were originally known as sarcoma growth factor (SGF) as they were produced by murine sarcoma virus-transformed cells (De-Larco and Todaro, 1978). Based on biological properties and biochemical characteristics, it was found that SGF activity actually came from two unrelated growth factors, transforming growth factor α (TGF-α) and transforming growth factor β (TGF-β). TGF-β has in multiple forms, for example, TGF-β1 and TGF-β2, and has been shown to inhibit a variety of immune responses. Wahl et al. (1988) reported that TGF-βs are potent inhibitors of IL-1-dependent lymphocyte proliferation and antigen-specific T cell proliferation and modulate MHC class II gene expression. As these immunosuppressive effects resemble that of cyclosporin, we tested the effect of two TGF-βs on

Control
CsA
Anti-CD3
Anti-CD3 + CsA

Fig. V.III.5. Cyclosporin A inhibits DNA fragmentation in thymocytes after *in vivo* administration of anti-CD3 antibody. Three-week-old Balb/c mice were injected i.p. with 200 µl of concentrated 145-2c11 supernatant. Animals also received 25 µg per g body weight CsA in 200 µl olive oil. Thymic DNA was analyzed 24 h after treatments. The observed effect of CsA plus anti-CD3 vs anti-CD3 alone was representative of 6 out of 8 mice treated.

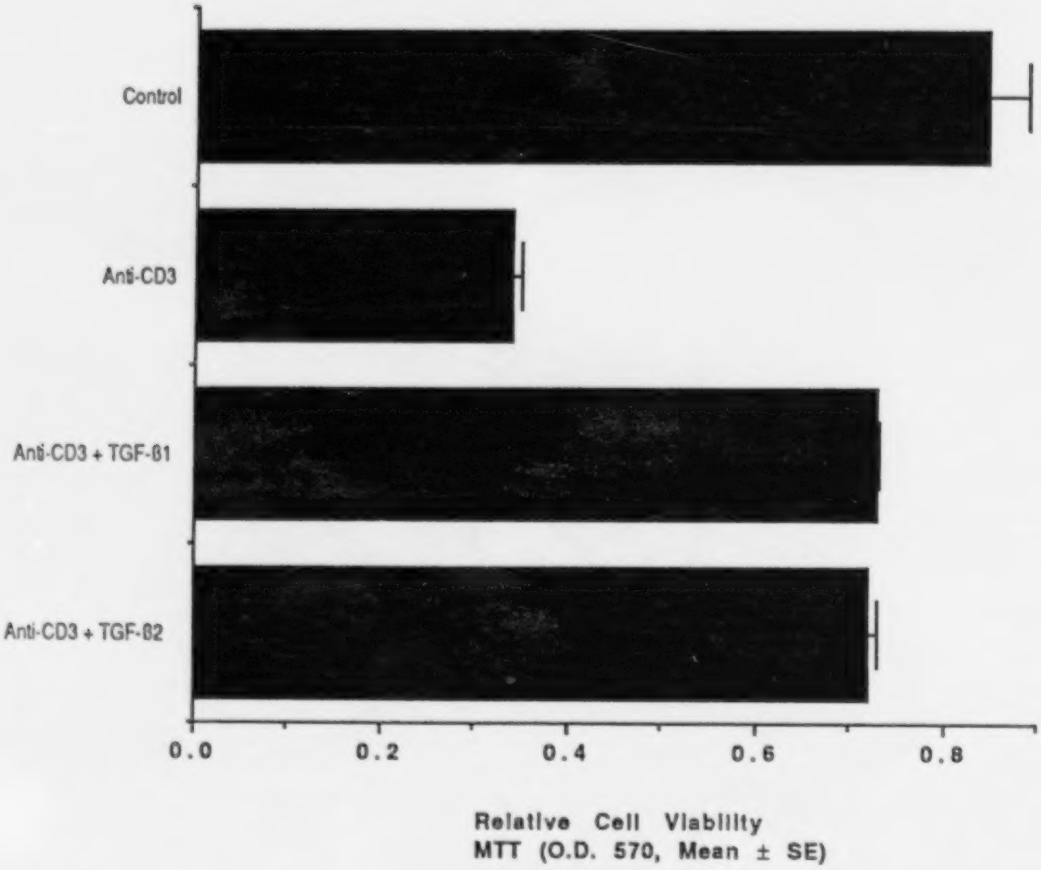


Fig. VIII.6. Inhibition of activation induced cell death in T cell hybridoma by TGF-β1 and TGF-β2. A1.1 cells (1 X 10^5) were activated with anti-CD3 coated plastic with/without 5 ng/ml TGF-β1 or TGF-β2 and compared to untreated controls. Cell viability was assayed by MTT at 12 h after stimulation.

activation-induced cell death. We activated T cell hybridoma A1.1 cells with anti-CD3-coated plastic tissue culture plates in the presence of TGF-β1 or TGF-β2 (5 ng/ml). As shown in Fig. VIII.6, both TGF-β1 and TGF–β2 blocked cell death in the T cell hybridoma as assayed by MTT. To examine the effect of TGF-β on activation induced DNA fragmentation, we activated ^{125}I-deoxyuridine labeled A1.1 cells with anti-CD3 coated plastic, in the presence of TGF-β1 and determined the percentage of DNA fragmentation 10 h later. TGF-β1 effectively prevented anti-CD3-induced genomic DNA fragmentation (Fig. VIII.7).

Altman et al. (1990) have demonstrated that TGF-β2 is present in mouse amniotic fluid (MAF) in association with α-fetoprotein. We tested the effect of MAF on activation induced cell death in our T cell hybridoma. As shown in Fig. VIII.8, 10% MAF inhibited activation induced cell death in A1.1 cells. This effect was neutralized by anti-TGF-β2 antibodies, while anti-TGF-β2 antibody alone did not show any effect on cell viability of the T cell hybridoma. Recent experiments by Tomasi et al. (personal communication) showed that a few injections of 3 ng of TGF-β2 in combination with α-fetoprotein prevented rejection of allografts in mice. Interestingly, when we injected concentrated MAF into mice, it inhibited anti-CD3 induced DNA fragmentation in the thymus *in vivo* (Fig. VIII.9). Because TGF-βs are expressed in many tissues, their regulatory effect on cell mortality may have important physiological implications. For example, the selective expression of TGF-β in medulla of the thymus (Ellingsworth et al., 1986) may contribute to the resistance of the medullary thymocytes to activation-induced cell death.

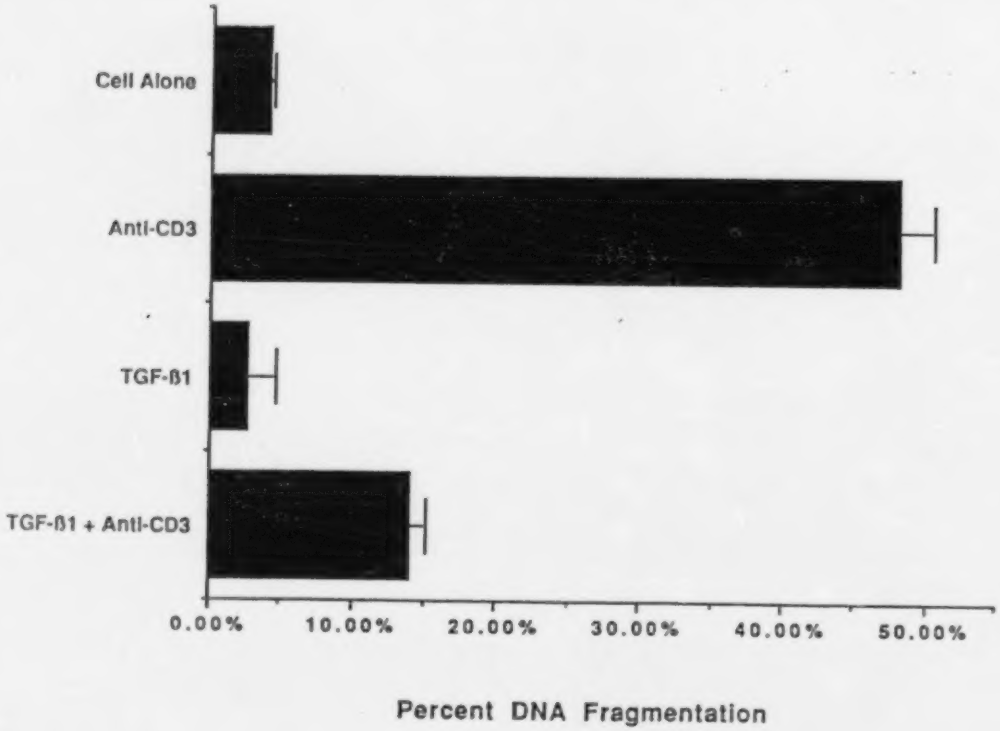


Fig. VIII.7. Inhibition of activation induced DNA fragmentation in T cell hybridoma A1.1 by TGF-β1. A1.1 cells were labeled with ^{125}IUDR for 10 h and activated on anti-CD3 coated plastic. Percentage of DNA fragmentation was determined at 10 h after anti-CD3 stimulation.

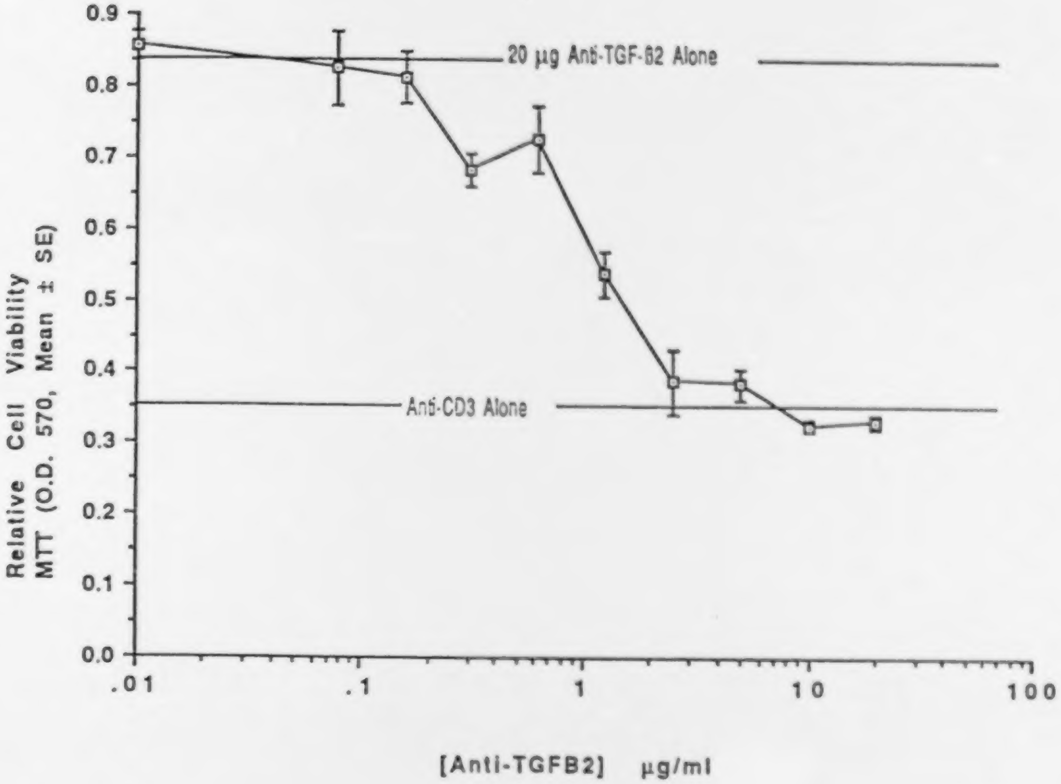


Fig. VIII.8. Anti-TGF-β2 neutralize the inhibitory effect of mouse amniotic fluid (MAF) on activation induced cell death. A1.1 cells were treated with anti-CD3 coated plastic and 10% mouse amniotic fluid in the presence of various concentrations of anti-TGF-β2 antibodies. Cell viability were assayed at 12 h post treatment. Anti-TGF-β2 neutralized the inhibitory effects of activation induced cell death of MAF.

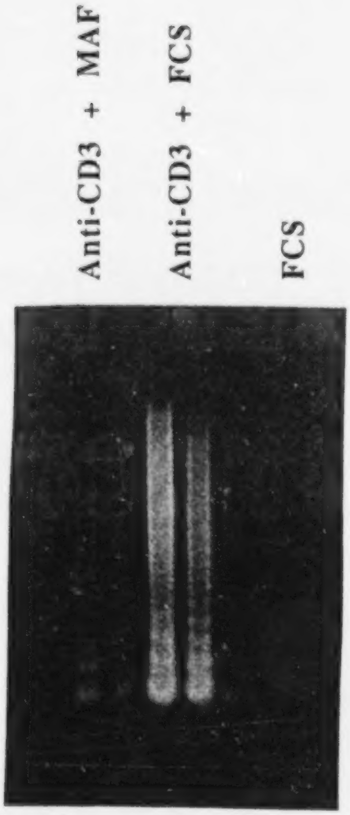


Fig. VIII.9. Inhibition of anti-CD3 induced DNA fragmentation in thymocytes in vivo by mouse amniotic fluid in vivo. 7 week old Balb/C mice were treated with anti-CD3 plus 0.15 ml of 100 X concentrated mouse amniotic fluid (MAF), 0.15 ml 5 X diluted fetal calf serum, or FCS alone. Total fragmented DNA was extracted at 8.5 h post anti-CD3. Two lanes for each treatment.

IX. POSSIBLE INVOLVEMENT OF *c-myc* IN ACTIVATION INDUCED CELL DEATH

As we have demonstrated and discussed, activation induced cell death, most probably the mechanism of negative selection during T cell development, is an active suicide process requiring RNA and protein synthesis. Increased production of several proteins was observed in dying thymocytes following treatment with glucocorticoids (Voris and Young, 1981). It strongly suggests that new products are synthesized during induction of apoptosis, and therefore there may be a genetic basis for apoptosis. However, most attempts to isolate the genes or the gene products responsible for cell death pathways have been unsuccessful (Waring, 1991). Nevertheless, it is well established that activation of T cells involves the expression of a number of genes, including early induction of several genes identified as protooncogenes. Unfortunately, the functions of protooncogenes are largely still to be characterized.

We attempted to determine whether protooncogenes play a role in activation-induced cell death. A number of investigators have employed antisense oligonucleotides to regulate cell function in a number of systems, including T cell activation (Gewirtz, et al., 1989) and T cell receptor expression (Zheng et al., 1989). We employed antisense oligodeoxynucleotides corresponding to a number of different protooncogenes (all 21-mer starting from the translation initiation codon, Table IX.1), and examined their effects on activation-induced cell death in our T cell hybridoma system. Various antisense oligodeoxynucleotides were added at 100 μM to A1.1 cell cultures with or without anti-CD3 treatment. Cell viability was assayed by MTT at 10 h after stimulation. Fig. IX.1 shows that anti-CD3 induced dramatic loss in cell viability in A1.1 cells, as previously demonstrated. Of the various protooncogene antisense oligodeoxynucleotides added to anti-CD3 treated A1.1 cells, only antisense to *c-myc* showed an inhibitory effect on cell death induced by anti-CD3 treatment. None of the

Table IX.1. Sequences of Antisense Oligodeoxynucleotides.

Name of the Antisenses	Sequence (From 5' to 3')
AS-*c-myb*	GTG TCG GGG TCT CCG GGC CAT
AS-*bcl-2*	TGT TCT CCC GGC TTG CGC CAT
AS-*c-abl*	CTT CAA ACA GAT CTC CAA CAT
AS-*c-fos*	GTT GAA ACC CGA GAA CAT CAT
AS-*c-myc*	GAA GTT CAC GTT GAG GGG CAT
S-AS-*c-fos*	ACC CGA GAA CAT CAT
S-AS-*c-myc*	CAC GTT GAG GGG CAT
S-NS-*c-myc*	AGT GGC GGA GAC TCT

AS: Antisense.

NS: Nonsense randomized sequence.

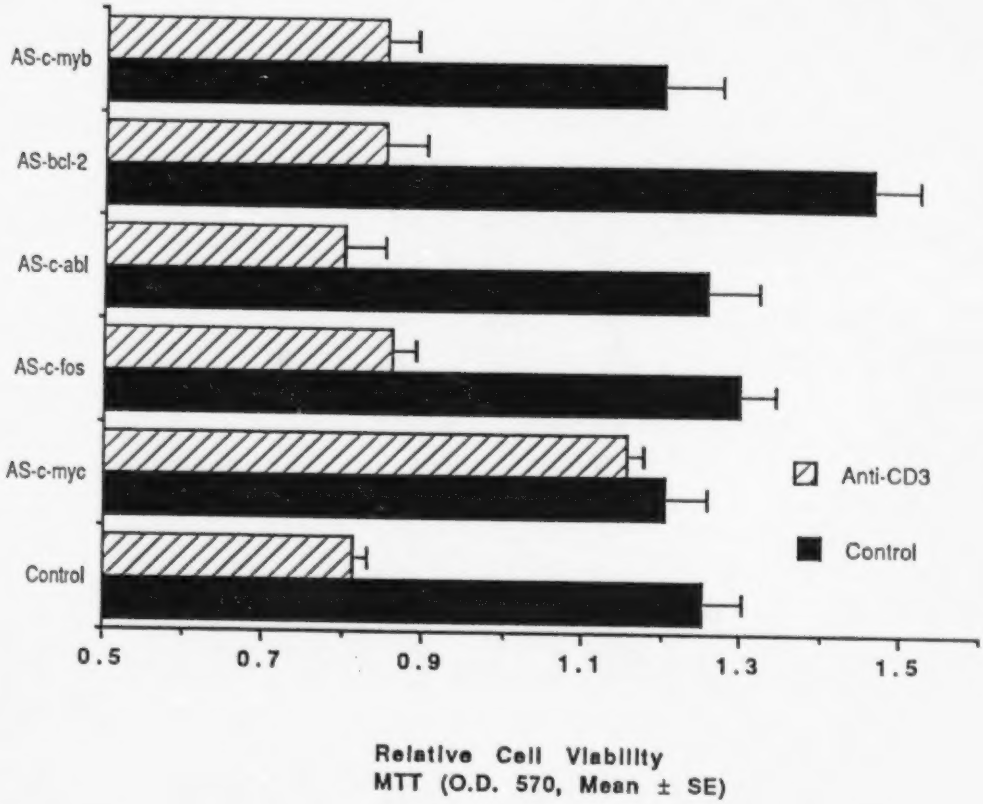


Fig. IX.1. The effect of antisense oligodeoxynucleotides corresponding to protooncogenes on activation induced cell death. A1.1 cells (10^5) were stimulated by anti-CD3-coated plastic in 100 µl of serum-free RPMI with 100 µM of various antisense oligodeoxynucleotides. Ten h after anti-CD3 stimulation, relative cell viability was assayed by MTT.

oligodeoxynucleotides showed toxicity to A1.1 cells at 10 h after addition as assayed by MTT. We concluded that the *c-myc* oncogene may play an important role in activation-induced cell death and therefore selected antisense to *c-myc* for further characterization. The effect of antisense to other protooncogenes were not tested further, except used as control oligos.

The effect of antisense oligodeoxynucleotides corresponding to *c-myc* on anti-CD3 induced cell death in the T cell hybridoma was also examined in DNA fragmentation assays. The genomic DNA of A1.1 cells was labeled overnight with ^{125}I-labeled deoxyuridine (^{125}IUDR). Six hours after treatment with anti-CD3, with or without the addition of antisense oligodeoxynucleotides to *c-myc*, cells were lysed with a buffer containing a non-ionic detergent which does not interfere with nucleosome structure. The fragmented DNA was separated from the intact DNA by centrifugation. The percentage of DNA fragmentation was obtained by assessing the ^{125}I in the supernatant and in the pellets. As shown in Fig. IX.2, the antisense oligodeoxynucleotides significantly reduced DNA fragmentation in A1.1 T cell hybridoma cells induced by anti-CD3 antibodies. This confirmed the results obtained with the MTT assay.

The effect of antisense oligodeoxynucleotides was sharply reduced at 12 h after addition (data not shown) and a relatively high concentration was needed. This might be due to the sensitivity of the phosphodiester bond in oligodeoxynucleotides to nuclease in assay cells and in the culture medium (Eckstein, 1985). Recently, researchers have used phosphorothioate oligodeoxynucleotides which contain a sulphur-for-oxygen substitution at the phosphate moiety. This substitution conserves the original charge and maintains high water solubility while providing resistance to nuclease (Eckstein, 1985). We therefore decided to use phosphorothioate oligodeoxynucleotides in our experiments. As shown in Fig. IX.3, we found that as little as 2.5 µM of the S-

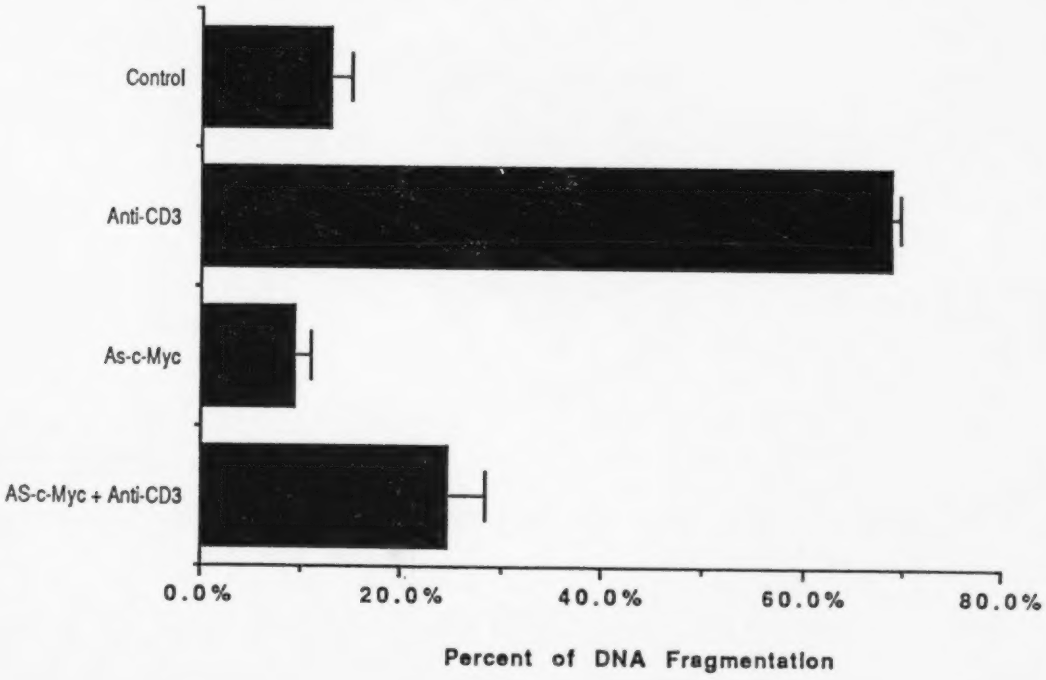


Fig. IX.2. Inhibition of activation-induced genomic DNA fragmentation by *c-myc* antisense oligodeoxynucleotides. A1.1 cells were labeled with ^{125}IUDR for 7 h. After 6 h incubation with 100 μM *c-myc* antisense (AS-c-myc), cells were treated with anti-CD3-coated plastic plus 50 μM *c-myc* antisense. DNA fragmentation was assayed at 6.5 h after anti-CD3 stimulation and presented as percentage of fragmented DNA over total DNA.

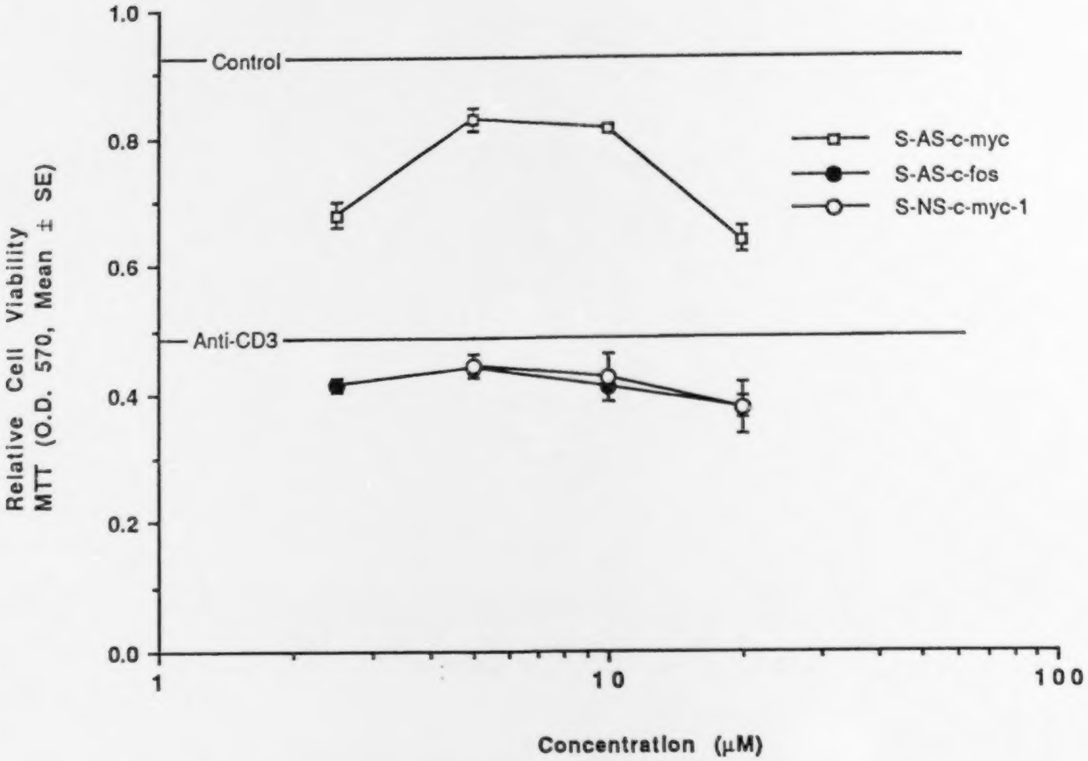


Fig. IX.3. Inhibition of activation induced cell death by *c-myc* antisense-phosphorothioate oligodeoxynucleotides. A1.1 cells (10^5) were stimulated with anti-CD3-coated plastic to which different concentrations of sulphur-modified antisense to *c-myc*, *c-fos* or random sequences with the same base composition as antisense to *c-myc* were added (sequences are shown in Table IX.1). Relative cell viability was assayed by MTT at 12 h post anti-CD3 stimulation.

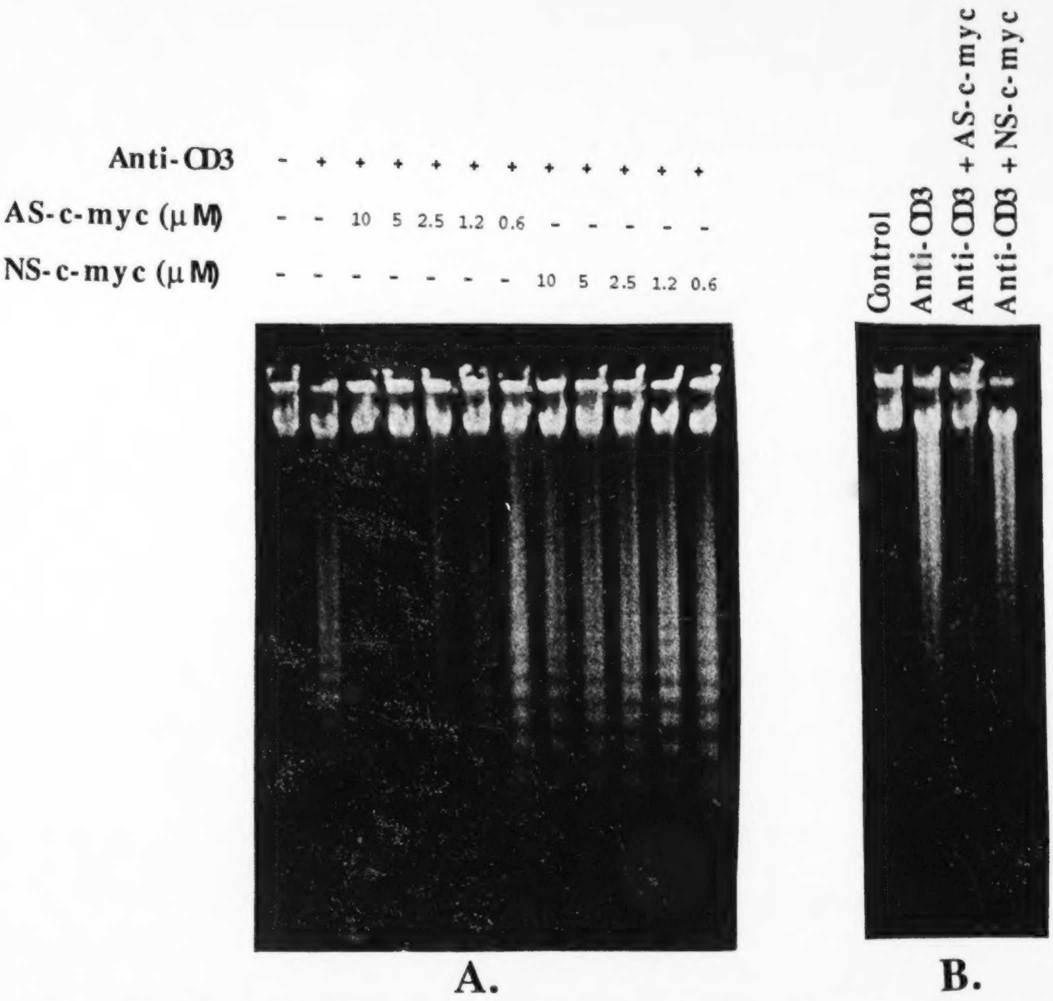

Anti-CD3 - + + + + + + + + + + +
AS-c-myc (μM) - - 10 5 2.5 1.2 0.6 - - - - -
NS-c-myc (μM) - - - - - - - 10 5 2.5 1.2 0.6
Control
Anti-CD3
Anti-CD3 + AS-c-myc
Anti-CD3 + NS-c-myc
A.
B.

Fig. IX.4. Inhibition of activation induced-genomic DNA fragmentation in T cell hybridomas. Cells were lysed in a lauryl sarcocinate containing buffer. After digestion with proteinase K and RNase A, DNA fragmentation was analysed directly on agarose gels. (A). A1.1 cells were stimulated with anti-CD3-coated plastic in the presence of different concentrations of phosphorothioate oligodeoxynucleotides corresponding to *c-myc* or a random sequence with the same base composition (NS-*c-myc*) (Seqences are shown in Table IX.1). DNA fragmentation was analyzed at 8.5 h after stimulation. (B). Genomic DNA analysis of T cell hybridoma IE6 cells treated with anti-CD3 with/without antisense to *c-myc* or the random sequence (5 μM).

substituted 15-mer of antisense to *c-myc* prevented cell death (as assayed by MTT), an effect not seen with antisense to *c-fos* or two random sequences with the same base composition of the *c-myc* antisense at any concentration tested. The S-substituted antisense to *c-myc* also blocked DNA fragmentation in a concentration-dependent manner, while no effect was observed with a random sequence at any concentration tested (Fig. IX.4A). In addition, antisense oligodeoxynucleotides to *c-myc* also blocked activation induced DNA fragmentation in another T cell hybridoma IE6 (Fig. IX.4B), indicating that this is a general effect not specific to A1.1 cells.

T cell hybridomas release lymphokines such as IL-2 following activation. In experiments to determine the effect of antisense to *c-myc* and the control oligodeoxynucleotide sequences on the production of CTL.L stimulatory lymphokines, we found no significant inhibition (Fig. IX.5), despite the ability of antisense to *c-myc* to inhibit cell death almost completely (Fig. IX.3). This accords with observations by others (Heikkila et al., 1987; Harel-Bellan et al., 1988) that *c-myc* antisense inhibits the entry into S phase of activated mature T cells, but not blast transformation or the expression of the IL-2 receptor in these cells, although both follow *c-myc* expression during T cell activation. These results indicate that the inhibition of activation induced cell death by *c-myc* antisense oligodexynucleotides is a specific effect rather than a general toxic effect. It also indicates that activation induced cell death and lymphokine production occur independently and have different genetic requirements, for example, during activation the expression of *c-myc* gene is not required for IL-2 production.
Antisense oligodeoxynucleotides corresponding to the protooncogene *c-myc* have been shown to inhibit *c-myc* protein expression and mitogenesis in human T cells (Heikkila et al., 1987; Harel-Bellan et al., 1988), to inhibit the growth of transformed B cells (McManaway et al., 1990), and to induce the differentiation of HL-60 cells (Holt et al., 1988). We demonstrated that both *c-fos*

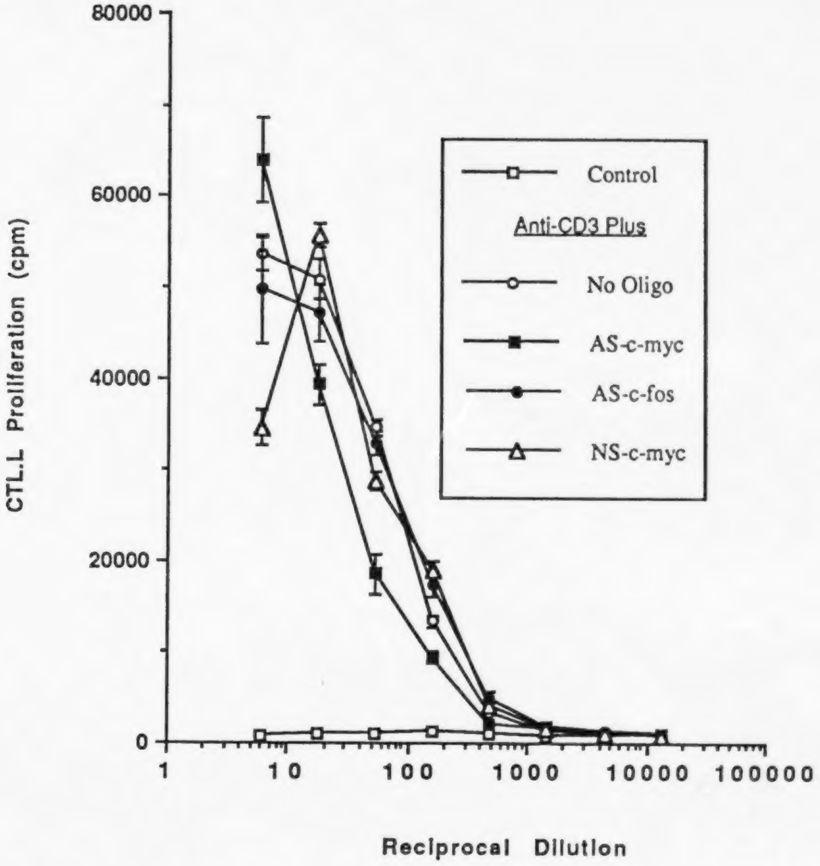


Fig. IX.5. The effect of phosphorothioate oligodeoxynucleotides on activation induced IL-2 production in T cell hybridoma. The supernatant samples were collected from the phosphorothioate oligodeoxynucleotides titration experiment (Fig. IX. 3). These supernatant samples were titrated and used for IL-2 assay with CTL.L cells. It shows that antisense to *c-myc* did not block anti-CD3 induced IL-2 production.

antisense, which could not prevent cell death, and *c-myc* antisense, which could prevent it, can block the proliferation of L929 cells, while a *c-myc* nonsense sequence had no effect on the growth of these cells (Fig. IX.6). When the level of *c-myc* protein was examined by immunoblot analysis with antiserum to a *c-myc* peptide, it was found that *c-myc* antisense phosphorothioate oligodeoxynucleotides specifically blocked its expression as compared with a scrambled *c-myc* sequence (Fig IX.7).

While *c-myc* antisense inhibits activation induced cell death in the T cell hybridoma, it has no effect on cell death induced by dexamethasone in the same cell line and another cell line, S49.1 (Fig. IX.8). Cell death induced by glucocorticoid is known to proceed differently from activation induced cell death; activation and glucocorticoid induced cell death actually act antagonistically (Zacharchuk et al., 1989). Such antagonistic effect was also observed in the A1.1 cells (Fig. IX.9). Interestingly, dexamethasone is known to inhibit *c-myc* expression (Yuh and Thompson, 1989), and our demonstration that *c-myc* antisense prevents cell death suggests that this inhibition may account for the ability of dexamethasone to inhibit activation induced apoptosis.

It has been demonstrated that RNA and protein synthesis inhibitors could prevent apoptosis of thymocytes induced by glucocorticoids (Wyllie, 1980) or calcium ionophore (McConkey et al., 1989), of T cells induced by growth factor withdrawal (Duke and Cohn, 1986) and of T cells hybridomas induced by activation (Ucker et al., 1989). It has been proposed based on these observations that apoptosis requires gene activation. However, McConkey et al. (1990) found that the treatment of thymocyte with RNA and protein synthesis inhibitors resulted a rapid loss in nuclear endonuclease activity. Thus, constitutively expressed gene products, such as *c-myc*, with a high turnover rate might play roles in apoptosis. We have found that *c-myc* is constitutively expressed in A1.1 T cell hybridoma, with no effects observed upon 4 hours of activation with anti-CD3 (Fig. IX.10). This indicates that *c-myc* expression is required but not sufficient for activation induced cell death.

How *c-myc* contributes to activation induced cell death is not known at present. Indeed that *c-myc* has any role to play in cell death is surprising, because it is usually implicated in cell cycle progression and cell transformation (Spencer and Groudine, 1991). It has been demonstrated that a cell line transfected with the *c-myc* gene controlled by a heatshock promoter underwent cell death (Wurms et al., 1986) and DNA fragmentation following heat shock (Bissonnette and Green, unpublished observation). A similar relationship between *c-myc* expression and apoptosis was described by Wyllie and colleagues (1987), who observed an increased rate of apoptosis in fibroblasts transformed with *myc* oncogenes. More recently, Evan et al. (1992) demonstrated that the expression of *c-myc* was responsible for apoptosis in low serum of c-myc transfected fibroblasts. It has been shown that *myc* protein specifically binds to nucleotide sequences in DNA (Blackwell et al., 1990) and, as a heterodimer with other proteins (Pendergast et al., 1991), may regulate the activity of other genes. The specific interaction of *myc* protein with the retinoblastoma (Rb) protein (Rustgi et al., 1991) and Rb has been demonstrated to regulate the transition from G1 to S phase (DeCaprio et al., 1989). It is thus of considerable interest to determine how *myc* controls activation induced cell death.

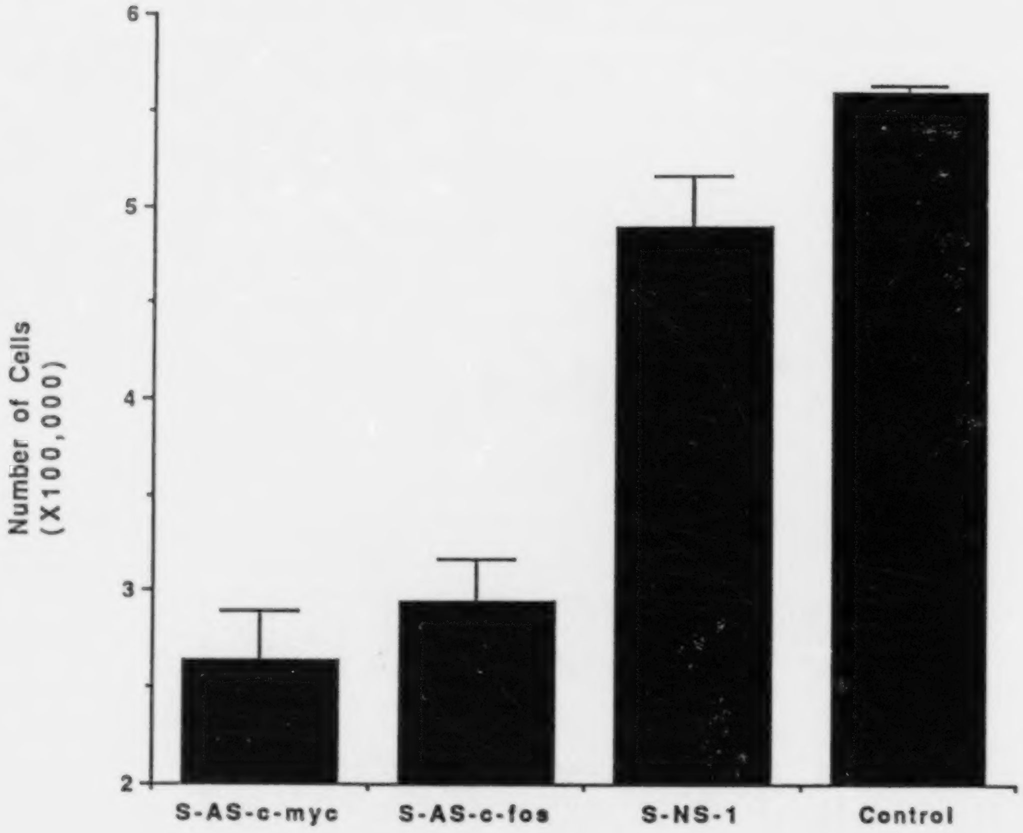


Fig. IX.6. Effect of phosphorothioate oligodeoxynucleotides on the proliferation of fibroblast L929 cells. L929 cells (2 x 10^5) were cultured in 1 ml IMDM suppelmented with 10% FCS in the presence of 2.5 µM oligodexynucleotides added at day 0, day 1 and day 2. On day 3, cells were harvested by treating with 530 µM EDTA + 0.05% trypsin and counted with a Coulter counter.

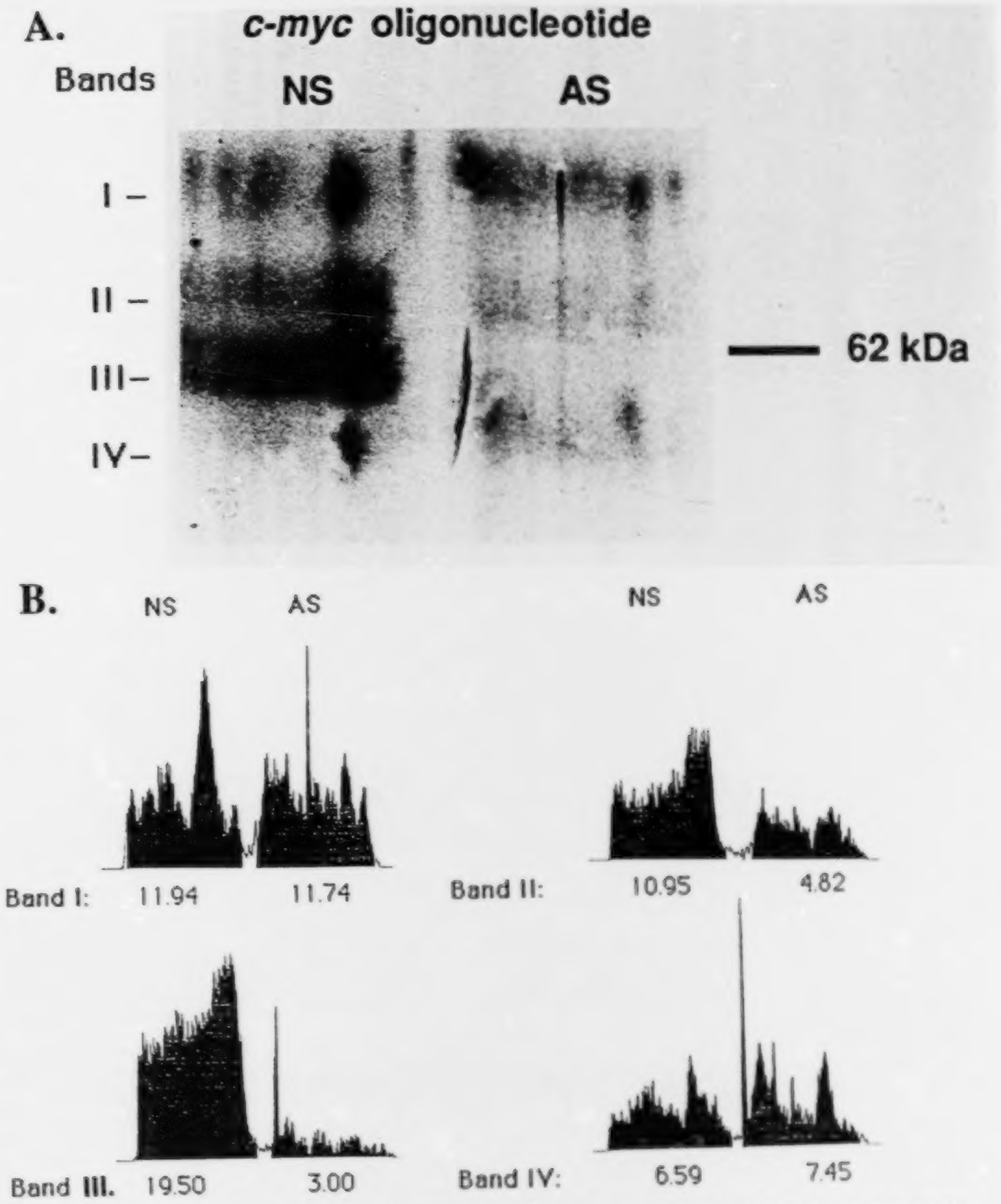

A.
c-myc oligonucleotide
Bands
NS
AS
I –
II –
III–
IV–
62 kDa
B.
NS
AS
NS
AS
Band I:
11.94
11.74
Band II:
10.95
4.82
Band III.
19.50
3.00
Band IV:
6.59
7.45

Fig. IX.7. Effect of oligonucleotides on *c-myc* protein expression. (**A**) A1.1 cells were cultured with antisense or nonsense *c-myc* phosphorothioate oligodeoxynucleotides (10 µM) for 18 h and cell lysates were Western blotted with a rabbit antiserum to a c-myc peptides (Ala-Pro-Ser-Glu-Asp-Ile-Trp-Lys-Lys-Phe-Glu-Leu-Leu). (**B**) Densitometric analysis of the immunoblot exposed X-ray film. The numbers represent the area of O.D.mm.

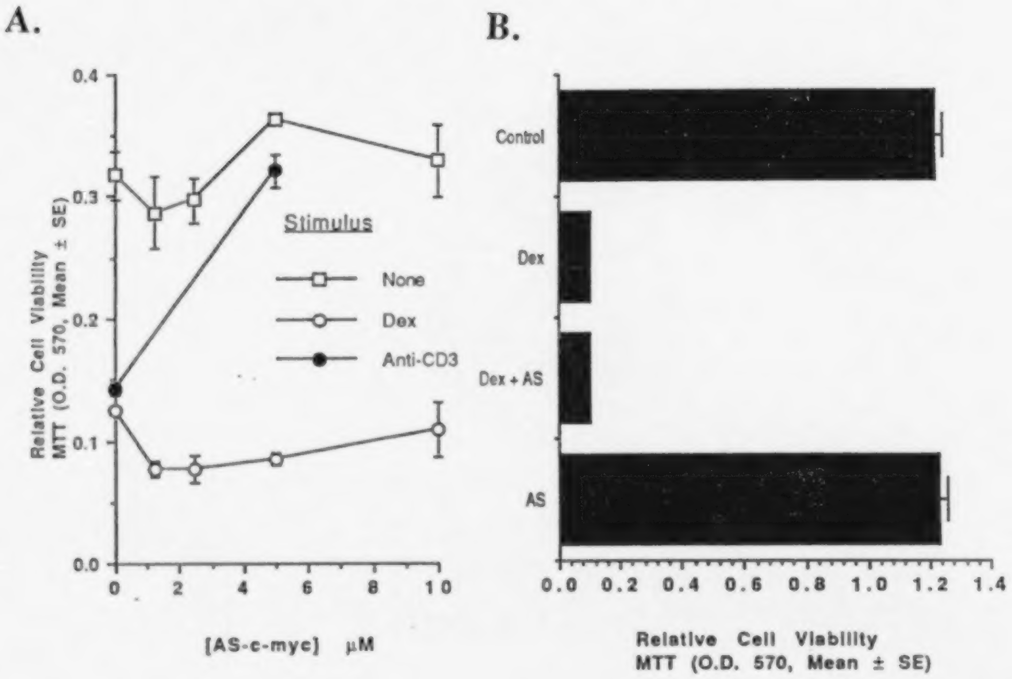


Fig. IX.8. The inability of antisense to *c-myc* to prevent cell death induced by glucocorticoid in T cell hybridoma and S49.1 cells. (A) 10^5 A1.1 were cultured with anti-CD3 or with 10^{-6} M dexamethasone with/without different concentrations of phosphorothioate oligodeoxynucleotides antisense to *c-myc*. Cell viability was assayed at 24 h after stimulation. (B) 10^5 S49.1 cells were cultured in the presence or absence of 10^{-6} M dexamethasone with 100 µM conventional *c-myc* antisense. Cell viability was assayed by MTT.

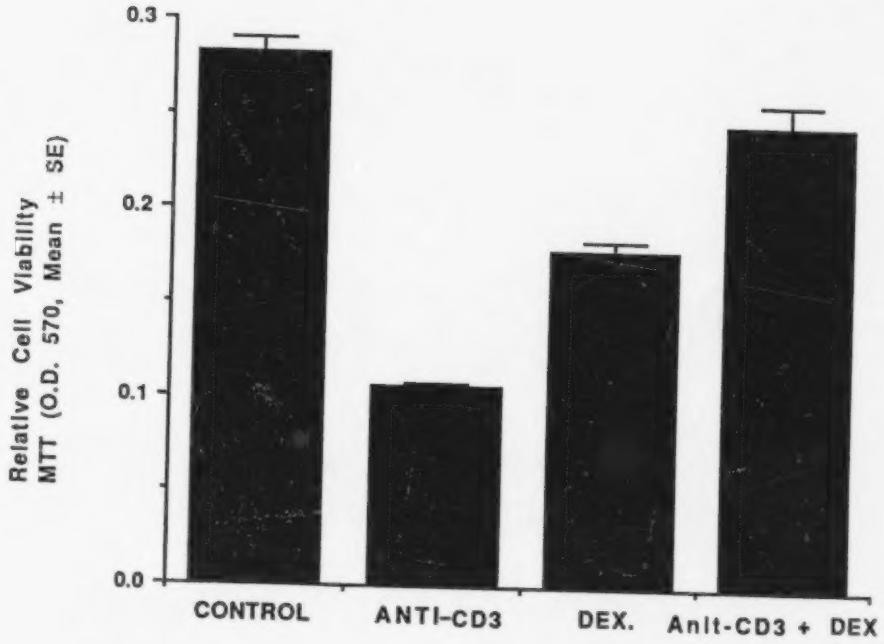
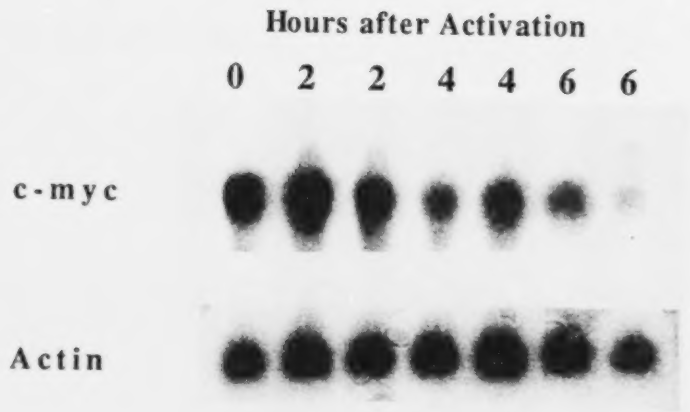


Fig. IX.9. Antagonistic effect of anti-CD3 and glucocorticoid on cell death in A1.1 cells. Cells (2 x 10^5) were cultured with anti-CD3, 10^{-6} dexamethasone, or both for 24 h. Cell viability was assayed by MTT. Nontreated cells are presented as control.


Hours after Activation
0
2
2
4
4
6
6
c-myc
Actin

Fig, IX.10. Northern blot analysis of *c-myc* expression. Total RNA was extracted from T cell hybridoma A1.1 cells at different times after activation with anti-CD3-coated plastic. The RNA samples were then transferred onto a nylon paper and hybridized with murine *c-myc* probe and β-actin probe.

X. DISCUSSION

A. Introduction

The thymus is constantly populated throughout the life of an individual by T cell precursors derived from the hemopoietic tissue. After arrival at the thymus, T cell precursors proliferate and undergo random rearrangement of their genes for antigen receptors. To generate a mature functional peripheral T cell repertoire, T cell precursors go through a number of processes in the thymus. One of these events is positive selection of self-MHC restriction, when maturing thymocytes are selected to recognize antigens in association with self-MHC proteins expressed in the thymus epithelial and dendritic cells (von Boehmer, 1991). Another process is deletion, or negative selection, of those cells with specificity for self-antigens. Negative selection has been considered an important process for establishing self-tolerance (Miller et al., 1990). Our knowledge about negative selection has expanded considerably in the last few years, due to the availability of monoclonal antibodies specific to different Vβ regions of the T cell receptors and to the development of T cell receptor transgenic mice (Blackman et al., 1990; von Boehmer and Kisielow, 1990). It has been demonstrated that T cells with specificity to self-antigens are not present in the mature T cell repertoire. However, it is not clear how autoreactive T cells are removed. The experiments described in this thesis were designed to address this problem. By *in vivo* administration of antibodies to the T cell antigen receptor complex, we have demonstrated that activation through the T cell receptors of immature T cells induces apoptosis.

Apoptosis is a programmed active self-destructive process, important in maintaining cellular homeostasis during development and in some diseases (Wyllie, 1987; Cotter et al., 1990; Cohen et al., 1992). However, the mechanism by which apoptosis takes place is largely unknown due to a lack of means of investigation. In order to understand the process of negative selection during T cell development, as well as the mechanism of apoptosis, we have developed an *in vitro* model system using T cell hybridomas, which can be induced to undergo apoptosis upon activation. Using this *in vitro* model we have demonstrated some of the mechanisms involved in activation-induced apoptosis. The following discussion is organized into topics according to the questions addressed in the results sections.

B. Activation-induced Cell Death in Immature Thymocytes *in vivo*

We demonstrated that 18 h after *in vivo* administration of antibodies to the CD3-TCR complex, the genomic DNA of thymocytes, but not that of mature peripheral T cells, was fragmented in the same fashion as that of cells undergoing apoptosis (Fig.III.4). By 40 h after injection of anti-CD3, nearly all of $CD4^+CD8^+$ and more than 50% of $CD4^+CD8^-$ thymocytes were eliminated, while there was no significant change in the total number of $CD4^-CD8^+$ and $CD4^-CD8^-$ thymocytes (Table III.3). Taken together, these results directly demonstrate that activation of thymocytes at immature stages during development in the thymus causes apoptosis, and this might play an important role in preventing maturation of autoreactive T cells.

It has been shown that anti-CD3 stimulates $CD4^+CD8^+$ double positive thymocytes to undergo apoptosis in *in vitro* thymic organ culture (Smith et al., 1989). Similarly, McConkey et al. (1989a) and Tadakuma et al. (1990) have found that stimulation of thymocytes in cell suspension *in vitro* with anti-CD3 antibody also leads to apoptosis in this double positive population of thymocytes. A self superantigen in association with I-E molecule is normally recognized by $CD4^+CD8^-$ $V\beta17^+$ but not $CD4^-CD8^+$ $V\beta17^+$ cells. However, both populations are absent from the periphery of $I\text{-}E^+$ mice. Fowlkes et al. (1988) showed that *in vivo* administration of anti-CD4 antibodies could prevent the deletion of $CD4^-CD8^+$ $V\beta17^+$ cells in $I\text{-}E^+$ mice, indicating that phenotypically immature $CD4^+CD8^+$ cortical thymocytes are the target of clonal deletion. These, and our observations, agree with the finding that double positive cells that bear T cell receptors capable of recognizing self-antigens are induced to undergo apoptosis upon contact with specific antigen plus MHC.

At different stages of T cell development, the $\alpha\beta$ T cell receptor serves different functions. The ligation of the receptor on mature T cells by processed foreign antigen associated with self MHC leads to proliferation and differentiation into functional effector cells (Weiss, 1989). Antibodies to the CD3-TCR complex also induce proliferation of mature T cells (Leo et al., 1987), but immature thymocytes do not proliferate in response to these antibodies (Finkel et al., 1987). These observations agree with our results that anti-CD3-induced cell death occurs only in the thymus, not in the peripheral lymphoid organs. It has been proposed that the interaction between $\alpha\beta$ T cell receptor on immature T cells in the thymus and self-MHC complexed with self-antigen peptides can lead either to the development of T cells with the capacity to recognize foreign antigen-self-MHC complex (positive selection), or the deletion of autoreactive T cells (negative selection). However, it is not known how this different response between mature and immature T cells occurs. Three possible explanations have been suggested for how a single receptor can perform three functions (Finkel et al., 1989): a) the engagement of the T cell receptor generates different intracellular signals at different stages of development; b) differences in cell physiology of T cells at different stages change the response to the same intracellular signal; or c) the microenvironment in which T cells locate determines the outcome of the receptor ligation. It is known that a large proportion of immature thymocytes express the $\alpha\beta$ T cell receptor ($\alpha\beta TCR^{low}$) but at a level 5-10 fold lower than that expressed on the mature thymocytes and peripheral T cells ($\alpha\beta TCR^{high}$) (Roehm, 1984). It also has been shown that superantigen stimulates the deletion of nearly all $\alpha\beta TCR^{high}$ but only 30-50% of $\alpha\beta TCR^{low}$ thymocytes (White et al., 1989). Therefore, there is a heterogeneity of function in the T cell receptor on immature T cells. Finkel et al. (1989) found that antibodies to the T cell receptor increased intracellular Ca^{++} in $\alpha\beta TCR^{high}$, and caused a lower increase in $\alpha\beta TCR^{low}$ cells. When anti-CD3 was used, both $\alpha\beta TCR^{high}$ and $\alpha\beta TCR^{low}$ cells responded with a large increase in intracellular Ca^{++} concentration. Because the signals from the TCR is transduced through CD3 (Klausner and Samelson, 1991), the difference in Ca^{++} influx in the above observation might suggest that the Ca^{++} mobilizing cascade distal from CD3 is the same in both $\alpha\beta TCR^{high}$ and $\alpha\beta TCR^{low}$ thymocytes. The difference is in the coupling of TCR and CD3. We found that anti-CD3 induced more cell deletion than did anti-TCR (Table III.4 and Fig.III.8). This might be a reflection of the *in vivo* difference. Our results and those of Finkel et al. (1989) help to define the maturational stages at which negative selection occurs and the possible difference in TCR and CD3 coupling at different stages during T cell development.

The results in Table III.5 show that the most dramatic effects of anti-CD3 or anti-TCR antibody administration are on the $CD3^{low}$ population. The $CD3^{intermediate}$ thymocytes are also susceptible to apoptosis induced by anti-CD3, but less susceptible to anti-TCR. If the population of thymocytes affected by anti-TCR is the same as that affected by antigen-induced negative selection, then this appears to conflict with the results of Guidos et al. (1990) who found that Mls-induced negative selection affects the $CD3^{intermediate}$ cells. However, it has been shown that the superantigen Mls interacts with MHC and T cell receptor differently from the way normal antigen peptides interact (Dellabona et al., 1990; Pullen et al., 1990). Also, it is possible that this reflects a difference in the site of action. It is known that Mls is present in the bone marrow derived cells of the thymus medulla, and therefore effects are seen only on contact with this site, when the expression of CD3 may be intermediate.

Hengarter et al. (1988) showed Mls-induced negative selection occurs as the cells enter the medulla, while data presented here show that anti-CD3 and anti-TCR induced cell death throughout the cortex. Recently Murphy et al. (1990) reported that *in vivo* administration of OVA peptide to the peptide specific T cell receptor transgenic mice induced apoptosis extending from the subcapsule to deep cortex area. After 33 days of treatment with the specific peptide, the cortex became acellular although the cellularity of the medulla was relatively normal. Similarly, our histological study demonstrated that cell death induced by *in vivo* administration of anti-CD3 antibody was restricted to the thymic cortex (Fiq.III.5).

Table III.4 shows that anti-CD3 induces deletion in double positive and CD4 single positive cells, but not in CD8 single positive thymocytes. The same observation was also made by Finkel et al. (1991, presented at Keystone Immune Tolerance Symposium) with an *in vitro* organ culture system. One possible explanation for the difference in susceptibility of CD4 versus CD8 single positive cells is that the developmental process for CD4 single positive cells which renders mature T cells resistant to activation-induced cell death occurs later than that for CD8 single positive cells. A similar finding by MacDonald and Lees (1990) showed that in Mls^{a+} mice the autoreactive $V\beta6^+$ thymocytes, but not non-autoreactive $V\beta8^+$ thymocytes in the $CD4^+CD8^-$ subpopulation, selectively died during short term culture, apparently as an outcome of autologous activation. In contrast, Tadakuma et al. (1990) found that apoptosis induced by phorbol ester or calcium ionophore A23187 affected double positive thymocytes and a subset of $CD4^-CD8^+$ thymocytes. This difference may be due to differences in the activation system. An understanding of intracellular signal transduction in relation to the induction of activation-induced cell death will help to clarify the effects of activation on different thymocyte populations.

Administration of anti-CD3 antibody suppresses the immune system, although the mechanism of this suppression is not clear (Hirsch et al., 1988). Our observation that *in vivo* administration of anti-CD3 antibodies induces apoptosis in the thymus could provide some explanation for the immunosuppressive effect of anti-CD3 antibodies. Rueff-Juy et al. (1989) injected anti-CD3 antibodies into neonatal mice within 18 h of birth, followed by 2-3 injections at one-week intervals. They screened the effect of anti-CD3 treatment 1-7 weeks after the last injection and found that the neonatal injections of anti-CD3 depleted T lymphocytes in the peripheral lymphoid organs, but not in the thymus. This seems to contradict our observation; however, it is possible that recovery from the depletion of thymocytes has occurred in Rueff-Juy's system by the time of examination, because thymocytes only require 3-6 days to differentiate from the stage at which they first express TCR to the stage of mature functional signal positive cells (Scollay, 1991). Hirsch et al. (1988) have observed that, whereas anti-CD3 depletes peripheral blood T cells, it also causes the expansion of T cells in the peripheral lymphoid organs. These results could be explained by the possibility that anti-CD3 cause the cells to activate and thus be recruited to the lymphoid organs. Our observation that anti-CD3 induces apoptosis in immature T cells could give only one explanation of anti-CD3 induced immunosuppression. We still do not know how anti-CD3 suppresses the function of mature peripheral T cells *in vivo*.

The effect of *in vivo* administration of anti-CD3 antibodies on thymocytes may not be direct, but rather be a secondary effect of inducing some mediators that in turn cause apoptosis. We have shown that *in vivo* administration of staphylococcal enterotoxin B induced DNA fragmentation in thymus (Fig.III.6). This result is in accordance with the *in vitro* experiments by Jenkinson et al. (1989), which show that the exposure of developing T cells to staphylococcal enterotoxin B induces specific apoptosis in only those cells bearing Vβ8, not those bearing Vβ6. It therefore indicates that the effect is specific to thymocytes, and is not the result of indirect and nonspecific secondary mediators.

The ability of antibodies to the T cell receptor complex administered *in vivo* to induce apoptosis in developing thymocytes provides evidence to support our hypothesis that negative selection proceeds via activation-induced cell death in immature T cells. This provides some explanation for the immunosuppressive effect of *in vivo* injection of antibodies to the CD3 complex.

Is negative selection alone sufficient to prevent autoreactivity? It is likely that many self-antigens are actually not expressed in the thymus and it is conceivable that T cells with specificity to those extra-thymic antigens may not be eliminated during the development in the thymus. Thus, tolerance to those antigens must be established by some peripheral mechanisms. There are several lines of evidence to support this notion. Intravenous administration of soluble foreign antigens could induce an unresponsive state of an animal to the secondary challenge of the same antigen in an immunogenic form (Mitchison, 1964). Other experiments suggest that T cells in normal animals are responsible for suppressing production of some autoreactive antibodies (Green et al., 1983). Induction of tolerance to auto-antigens can also be achieved through the induction of anergy, during which, in the absence of sufficient secondary signals from antigen presenting cells, activation through the T cell receptors causes T cells to become impotent upon further stimulation (Schwartz, 1990). Interestingly, peripheral T cells in transgenic mice that were expressing an allogenic MHC class I antigen driven by an insulin promoter were anergic to islet cells (Moranhan et al., 1989). This unresponsiveness could be turned off *in vitro* by incubation with IL-2. The maintenance of peripheral tolerance can also be achieved by peripheral deletion. Kawabe and Ochi (1991) demonstrated that *in vivo* administration of SEB to BALB/c mice resulted in deletion of $V\beta8^+$ $CD4^+$ peripheral T cells. Similarly, Webb et al. (1990) showed that *in vivo* exposure of Mls^a antigen lead to specific disappearance of $V\beta6^+$ T cells. Whatever the mechanism involved, tolerance to autoantigens in the peripheral mature T cell repertoire has to be maintained.

C. Similarity Between Activation-induced Cell Death in T Cell Hybridomas (*In vitro*) and In Immature Thymocytes (*in vivo*)

Activation of mature peripheral T cells results in the production of lymphokines and entry into the proliferative stage of the cell cycle (Weiss, 1989). T cell hybridomas share properties of mature peripheral T cells and immature thymocytes. The ability of the T cell hybridomas to secrete lymphokines after engagement of the T cell antigen receptor with activation stimuli provides a convenient model for studying activation of T cells (Kappler et al., 1981; Fotedar et al., 1986). Activation of T cell hybridomas results in lymphokine production and, paradoxically, inhibition of their spontaneous growth (Ashwell et al., 1987). We have attempted to determine whether these cells undergo apoptosis upon activation resembling activation-induced cell death in immature thymocytes (as demonstrated in this thesis and Smith et al., 1989), and thus provide a model with which the mechanism of negative selection in the thymus can be elucidated.

We examined activation of a poly-18 specific, I-A^{d} restricted T cell hybridoma, A1.1, and found that cells undergo activation-induced cell death. Following activation of the hybridoma with antigen or other activating signals, the first signs of cell death can be detected at approximately 7 h, at which time membrane blebbings first appear and cells fail to exclude fluorescein (Fig.IV.2). DNA fragmentation was first detected at approximately 5 h post-activation as determined by agarose gel electrophoresis (Fig.IV.7) or 4 to 5 h post activation as determined by a fragmentation assay with radio-labeled cells. At 10 to 12 h, some of the cells fail to exclude Trypan blue. Ultrastructurally, many of the cells show nuclear chromatin condensation, but cytoplasmic organelles remain identifiable, even as late as 14 to 16 h post-activation. These ultrastructural changes were not observed in cells treated with sodium azide, which induces necrosis (Fig.IV.5). We also observed that the early stages of activation-induced cell death, including fluorescein uptake and DNA fragmentation, are inhibited by CHX and Act D (Fig.IV.9). These results strongly suggest that activation-induced cell death via apoptosis requires protein synthesis, and thus is an active suicide process.

These characteristics of activation-induced cell death in the T cell hybridoma are similar to the cell death observed in immature thymocytes induced by glucocorticoids (Wyllie, 1980; Cohen and Duke, 1984) and ionizing radiation (Sellins and Cohen, 1987) or in target cells killed by cytotoxic T cells (Duke et al., 1983). The T cell hybridoma was created by fusion of a mature T cell and a thymoma. The thymoma partner may represent a T cell developmental stage that contributes to the property of activation-induced cell death. This property may resemble activation-induced cell death in immature thymocytes, which has been proposed as a mechanism of elimination or negative selection of thymocytes that recognize self determinants during development in the thymus.

Two other groups have reported results which parallel those discussed here. Ucker and colleagues (1989) have shown that activation of the T cell hybridoma 2B4 results in cell death and DNA fragmentation. Activation-induced cell death shares many features in common with that induced in other ways. Cell death and DNA fragmentation were observed upon stimulation of a human T leukemia line via CD3 (Takahashi et al, 1989). Further, Mercep and colleagues (1989) have demonstrated that the ability of anti-CD3 to stimulate activation-induced cell death in T cell hybridomas depends upon the presence of the CD3 $\zeta\eta$ heterodimer on the cell surface. However, it is still not known why T cell hybridomas die in response to the same type of stimulation that would otherwise cause proliferation of mature T cells. It is possible that such activation-induced cell death is a peculiarity of their transformed phenotype. However, these and our results suggest that the phenomenon of activation-induced cell death in transformed T cells may be relatively common. Further studies with these systems will reveal useful information about apoptosis as it relates to the immune system development, cellular homeostasis, and immune responses.

The few *in vitro* model systems of apoptosis that currently exist include death of thymocytes induced by glucocorticoids, calcium ionophores, and low dose γ-irradiation. Smith et al. (1989) demonstrated that treatment of thymic organ culture with anti-CD3 induced massive DNA fragmentation and death of immature (especially $CD4^{+}CD8^{+}$ double positive) T cells. We have demonstrated a similar phenomenon in mice treated with anti-CD3 antibodies *in vivo*. We suggest that the cell death process in the T cell hybridoma cells triggered by TCR-CD3 complex-specific antibodies represents activation-induced cell death during negative selection. However, we do not have direct evidence that allows us to conclude that cell death in two systems us the same mechanism (see Table X.1 for comparison of their properties). In the absence of reproducible *in vitro* thymocyte culture models, T cell hybridoma cells are currently the best model representing activation induced apoptosis in immature thymocytes.

Our model with T cell hybridomas, because of the homogeneous nature of the cell population and physiologically related activation pathway, may prove to be an extremely useful *in vitro* model system for the analysis of the mechanism of apoptosis and negative selection during T cell development, with implications for self tolerance. We have thus carried out a series of experiments with this model to delineate the biochemical events involved in the processes of activation-induced cell death .

D. Possible Mechanisms of Activation-induced Cell Death in T Cell Hybridomas

We have demonstrated that activation of T cell hybridomas, like that of immature thymocytes *in vivo*, results in cell death. There are several possible explanations for such activation-induced cell death. It is probable that the activation signals directly

Table X.1. Comparison of Activation Induced Apoptosis in T Cell Hybridomas In Vitro and That in Immature Thymocytes In Vivo.

Features	In Vitro	In Vivo
Membrane Changes	+	+
Chromatin Condensation	+	+
Genomic DNA Fragmentation	+	+
Lymphokine Production	+	?
Cellular Homogeneity	+	-
Removal of Apoptotic Cells	-	+
Inhibited by CsA	+	+
Inhibited By ATA	+	+
Inhibited By AS-myc	+	?

activate intracellular autonomous destructive pathways. Alternatively, activation of these cells might stimulate production of some extracellular signals which then act on bystanders to cause them death. It is also possible that the receptors of some newly secreted extracellular mediators are expressed or upregulated upon activation. We have considered these possibilities and concluded that even though extracellular factors such as TNF were produced from activated A1.1 cells, unless these extracellular factors are very labile, it is unlikely that extracellular factors are responsible for initiating the cell death of bystanders in culture after activating stimulation.

Liu and Janeway (1990) demonstrated that γ-interferon played a critical role in Th1 cell death induced by anti-TCR/CD3 antibodies in the absence of costimulatory signals from accessory cells. It is possible that γ-interferon also plays a important role in our system. However, in our cell titration experiment, at all the cell densities tested, we were unable to demonstrate a difference in the percentage of cell death. This indicates that factors, like γ-interferon, are not responsible for activation-induced cell death in the T cell hybridomas. However, we cannot exclude the possibility of the expression of receptors for highly potent extracellular mediators, because the lowest cell density tested was 390 cells per well of 96-well plate.

Thus, we have demonstrated that activation-induced cell death of the T cell hybridoma proceeds through an autonomous process. It is hypothesized that activation signals through the T cell receptor-CD3 complex somehow trigger some of the key intracellular events leading to irreversible cell death pathways. At present, we still do not know how such an autonomous process happens. Further experiments performed with our T cell hybridoma system was to explore the biochemical processes involved in activation-induced cell death.

Cell death by apoptosis is a programmed event that does not cause damage to the surrounding cells *in vivo* (Wyllie, 1987). The total number of cells in an organism is kept constant by maintenance of a fine balance between cell death and cell proliferation (Cotter et al., 1990). Scientists have amassed considerable knowledge at both molecular and cellular levels about how cells divide. However, detailed information on the fine tuning of cell death is not available. Cell death is not only involved in cell homeostasis but also in some physiological processes and diseases. Hence, elucidation of the mechanism of cell death could enhance our understanding of some physiological processes, such as the deletion of immature thymocytes bearing inappropriate specificities, and in turn help us to control some diseases.

E. The Role of Nuclease in Activation-induced Cell Death

Nuclease activity leading to DNA fragmentation during apoptosis may depend either on the expression of an endonuclease gene product or on activation of pre-existing endonuclease after apoptosis induction. Cytotoxic T lymphocytes (CTL) induce DNA fragmentation in their target cells, but the target cells do not require the synthesis of macromolecules (Duke et al., 1983). It has been suggested that CTL transfer molecules that mediate a suicide pathway into the target (Henkart, 1985). Nucleases are probably not transferred from cytotoxic effector to target cells, rather Tian et al. (1991) reported that DNA fragmentation in permeablized thymocytes could be induced by a poly(A) binding protein-related granule protein, which by itself did not have nuclease activity. Similarly, Shi et al. (1992) purified a protein called fragmentin from NK granules that also induced DNA fragmentation upon introduction into target cells by perforin, though fragmentin itself did not possess nuclease activity. The occurrence of DNA fragmentation in isolated noninduced nuclei in the presence of Ca^{++} (Cohen and Duke, 1984) strongly suggest that the nucleases responsible for DNA degradation are present in noninduced cells. Therefore, activation of these nucleases during apoptosis induction may be a key event. The nucleases may be activated by removal of an inhibitory element by a protease, because protease inhibitors have been shown to prevent cell death (Shi et al., 1992; Y. Shi, C. Helgason, D.R. Green and C. Bleackley, unpublished observation), or by release from compartmentalized storage, or simply by an increase of Ca^{++} concentration in the nucleus (McCkonkey et al., 1989c). However, the last possibility is unlikely, because Lennon at el (1992) did not detect Ca^{++} increase in nuclei during apoptosis.

Genomic DNA fragmented into a nucleosome-sized ladder, an indication of endonuclease activity, has been considered a hallmark of apoptosis. However, the relationship between DNA fragmentation and cell death has not been well established. Based on observations of the kinetics of activation-induced cell death in T cell hybridoma, Odaka et al. (1990) reported that DNA fragmentation preceded cell death and suggested that it was a prerequisite, rather than a consequence, of cell death. In contrast, Vukmanivic and Zamoyska (1991) suggested that, in an activated T cell hybridoma, cell death was caused by failure of mitochondria, not fragmentation of genomic DNA. Thus, it remains to be clarified whether DNA fragmentation is an initiator of cell death.

ATA is a known nuclease inhibitor, which interacts with the nucleotide binding sites on nucleases and thus exerts an inhibitory effect on these enzymes (Hallick et al., 1977). It has been shown that including ATA in the buffer for RNA isolation from tissue can increase the yield of RNA (Skidmore and Beebee, 1989). We performed experiments to show that ATA could inhibit activation-induced DNA fragmentation and cell death in T hybridoma A1.1 cells *in vitro* (Fig. VI.2 & 3) and in thymocytes *in vivo* (Fig. VI.9). This effect does not appear to depend on altering major cellular metabolic pathways, since we could not demonstrate significant effects of ATA on DNA, RNA and protein synthesis (Fig. VI.4). Therefore, it is likely that ATA prevents both DNA fragmentation and cell death by inhibiting endonuclease activity, which suggests that DNA fragmentation initiates activation-induced apoptosis. Such a conclusion agrees with McConkey et al. (1989c), who reported that ATA could also interfere with glucocorticoid and

A23187 induced cell death in thymocytes. Batistatou and Greene (1991) reported that ATA could rescue PCl2 cells and sympathetic neuron cells from apoptosis caused by deprivation of nerve growth factors. Therefore, ATA might be a general inhibitor of apoptotic cell death.

Kinetic experiments also suggested that ATA interferes with apoptosis via its action on endonucleases (Fig. VI.6). The ability of ATA added at different times after activation to inhibit ultimate DNA fragmentation and ultimate cell death (as determined by MTT assay) strongly correlated with the extent of DNA fragmentation at the times of ATA addition. There was no correlation between ultimate cell death and cell death at those times. However, this conclusion contradicts the finding of Vukmanovic and Zamoyska (1991), who reported that the primary effect of ATA was to prevent mitochondrial failure rather than DNA fragmentation in anti-CD3 induced apoptosis of T cell hybridomas. It is interesting to note that upon activation with anti-CD3, one hybridoma used in their experiments did not lose the ability to cleave MTT but still showed fragmented genomic DNA and died. Therefore, functioning mitochondria, as detected by MTT, still cannot rescue cells from dying. In addition our experiments showed that loss of mitochondrial function follows DNA fragmentation, and that ATA prevents the loss of mitochondial function by inhibiting DNA fragmentation. Once DNA fragmentation has occurred, ATA is unable to prevent cell death. Our experiments strongly suggest that ATA exerts its effect on apoptosis by direct action on nuclease, indicating that DNA fragmentation is not the consequence but rather the prerequisite of cell death. Results obtained from a collaborative study with Dr. R.C. Bleakley and colleagues (submitted for publication) revealed that ATA could also prevent CTL-induced DNA fragmentation and death of target cells.

Recently, Brune et al. (1991) showed that spermine, which modifies chromatin arrangement and thus prevents endonuclease activity, could prevent apoptosis of thymocytes, thus indicating an indirect link between endonuclease activation and apoptosis. English et al. (1989) followed the events of DNA fragmentation and morphological changes in rat ventral prostate cells at different times after castration. They found that elevation of the $Ca^{++}Mg^{++}$-dependent endonuclease activity and fragmentation of genomic DNA were initiated long before cells were morphologically dead. Thus, DNA degradation must not be the consequence of cell death but instead occurs early in the chain of causal events.

Similar to many other pharmacological reagents, ATA possesses many other activities. It has been shown that ATA is a translation initiation inhibitor as detected in a hamster brain cell-free system (Nelson and Winkler 1987 and Theriaul and Gauthier, 1991). It also inhibits several types of DNA and RNA polymerases in *in vitro* systems (Nakane et al., 1988). Interestingly, it has been shown that at low concentrations, ATA could stimulate RNA and protein synthetic activities of the mitochondrial lysate (Kulkarni et al., 1987). These experiments were all performed in cell free systems, it is very likely that ATA activities might be different in intact cells.

As an aside to our discussion it should be pointed out that ATA has been shown to be a potent and selective inhibitor for HIV replication *in vitro* (Balzarini, 1986). It can also help the survival of HIV infected cells (Baba et al., 1988). This ability has been attributed to the inhibitory effect of ATA on the reverse transcriptase (Baba et al., 1988). Another mechanism by which ATA inhibits HIV infection is by the direct specific binding of ATA to the virus receptor-CD4 (Schols et al., 1989). Our finding that ATA inhibits apoptosis could be the third mechanism for the anti-HIV effect, as it has been shown that HIV virus induced apoptosis in T lymphoblasts (Terai et al., 1991). Thus, ATA has good potential for use as an anti-HIV drug. More studies on this interesting chemical might pave the way of the application of anti-HIV effect of ATA in clinical situations.

F. Signal Transduction and Activation-induced Cell Death

The involvement of signal transduction in apoptosis induction has been indicated in several cases. It has been shown that a sustained increase in cytosolic Ca^{++} level is associated with the activation of nuclease and subsequent cell death in thymocytes stimulated with glucocorticoid (McConkey et al., 1989d), or anti-CD3 antibodies (McConkey et al., 1989b). Our observation of the requirement of Ca^{++} in the culture media (Fig. VII.1 & 2) support the view of the involvement of Ca^{++} in programmed cell death. The experiment with isolated nonstimulated nuclei suggested that Ca^{++} is involved in the activation of endonuclease (Fig. VI.7). Some Ca^{++} dependent nucleases have been partly purified, for example, from thymocytes after induction of apoptosis (Wyllie et al., 1988). Vanderbilt et al. (1982) found that DNA autodegradation in isolated rat liver nuclei is dependent on the concentration of Ca^{++}, DNA fragmentation being enhanced at lower Ca^{++} concentrations and decreased at higher concentrations. It thus appears that an optimum calcium concentration is required for activation of nuclease. In some types of cell death such as those induced by TNF, the fragmentation of genomic DNA does not require intracellular or extracellular Ca^{++} (Haseqawa and Bonavida, 1989). This may indicate that TNF-induced cell death may occur by a pathway independent of induction of apoptosis by the triggering of Ca^{++} dependent nucleases. McConkey et al. (1989c) reported that ionophore A23197 could induce thymocyte death, suggesting that the sustained increase in intracellular Ca^{++} alone is able to mediate cell death. Our data and that of Mercep et al (1989) showed that elevation of intracellular Ca^{++} by ionomycin alone is not sufficient to cause T cell hybridoma death. Ionophore A23187 acts differently from ionomycin, because it has been shown that this ionophore alone is able to elevate intracellular Ca^{++} and activate protein kinase C (Dr. A. Ho, personal communication).

Activation of mature T cells by mitogenic stimulation involves degradation of membrane phosphotidalinositol 4,5-biphosphate, giving a transient increase in the production of DAG and IP3 (Manger et al., 1987). DAG causes translocation of PKC from the cytosol to the membrane-associated compartment, while IP3 increases the intracellular Ca^{++} concentration. Mature T cells respond to such activation with proliferation, whereas immature T cells or T cell hybridomas activated by the same type of stimuli often undergo apoptosis. Therefore, it is possible that these second messengers which normally promote growth are present in immature T cells or T cell hybridomas, but here cause cell death rather proliferation. Thus, both PKC and Ca^{++} might play important roles in activation-induced cell death. We have shown that the diacylglycerol analogue OAG, which stimulates PKC, induces apoptosis in A1.1 cells when added together with ionomycin (Fig. VII.3). Further, we have shown that the inhibition of PKC by staurosporin, or H-7, could prevent activation and inhibit anti-CD3-induced apoptosis in the T cell hybridoma (Fig. VII.5). This indicates that PKC is involved in activation-induced cell death. When we blocked PKC function by preincubating cells with high concentrations of PMA, we again were able to show that cell death was prevented (Fig. VII.6). Because PMA is specific to PKC, we conclude that PKC is likely involved in activation-induced cell death in T cell hybridomas. The evidence for the involvement of PKC in activation-induced cell death is controversial. Ojeda et al. (1990) reported that inhibition of PKC by H-7 inhibited cell death in thymocytes induced by glucocorticoids. On the other hand, McConkey et al. (1989b) reported that thymocytes appeared to undergo activation-induced apoptosis only in the absence of PKC activation. For example, DNA fragmentation was observed in thymocytes treated with concanavalin A and the PKC inhibitor H7, but not by con A alone (McConkey, et al., 1989). Similarly, McConkey et al (1990) observed that apoptosis in thymocytes induced with A23187 or anti-CD3 is inhibited by the addition of phorbol esters or IL1, both of which induce PKC. Furthermore, McConkey et al. (1989b) demonstrated that PMA could directly inhibit Ca^{++} dependent DNA fragmentation in isolated nuclei, suggesting that activation of PKC could inhibit Ca^{++} dependent endonuclease activity. It appears that the role of PKC can vary from system to system.

The contradicting results about PKC obtained by different investigators with different systems may be owing to the involvement of other kinases such as tyrosine kinases. It is possible that the PKC inhibitors employed might also have affinity to other kinases. In fact, it has been shown that staurosporin could inhibit tyrosine kinases (Ohmichi et al., 1992). Therefore, the exact role of PKC in induction of apoptosis still remain to be further defined.

G. Inhibition of Activation-induced Cell Death by Cyclosporin A and Transforming Growth Factors

Cyclosporin A is a widely used immunosuppressive drug in organ transplantations and autoimmune diseases. However, in some human recipients of organ transplants, a graft-versus-host disease condition develops (Hood et al., 1987). In animal models, injection of cyclosporin A for 7 days into neonatal mice induced autoimmunity in the thyroid, pancreas, salivary glands, gonads and stomach (Sakaguchi and Sakaguchi, 1989). Cyclosporin A treatment of syngeneic bone marrow reconstituted irradiated rats cause them to develop lethal graft-versus-host disease after cyclosporin A withdrawal (Glazier et al., 1983). The development of autoimmunity in these cases is thought to be related to the interference of cyclosporin A with clonal deletion of autoreactive T cells in the thymus. Many explanations have been suggested for such interference, including the downregulation of the class II expression on the thymic epithelia (Cheney and Sprent, 1985), or the effect on development of suppressor cells that regulate autoreactive T cells (Sakaguchi and Sakaguchi, 1989). Our observation that cyclosporin A could inhibit activation-induced cell death (Fig. VIII.2 & 5) suggests an alternative mechanism of cyclosporin A induced autoimmunity, that is, by direct interference with the intrathymic deletional process. Cyclosporin's ability to interfere with negative selection and to inhibit activation-induced cell death provides indirect evidence for the hypothesis that negative selection occurs by a process of activation-induced cell death.

Interference with T cell development in cyclosporin A induced autoimmunity was first directly shown by removing the thymus to eliminate the effect (Sorokin et al, 1986). Several studies have focused on the effects of cyclosporin A on T cell development. Jenkins et al. (1988) showed that CsA treatment prevented the deletion of Vβ17$^+$ T cells in I-E$^+$ mice. Gao et al. (1988) showed that cyclosporin A could block the differentiation of CD4$^+$CD8$^+$ cells into mature single positive cells and Vβ11$^+$ T cells developed normally in CsA teated I-E$^+$ B6 mice. These results clearly demonstrated that CsA could interfere with negative selection process. Our observation of the inhibition of activation-induced cell death by CsA might be the mechanism of its effect on negative selection, as we have shown that negative selection proceeds through activation-induced cell death.

The mechanism by which cyclosporin A exerts its effect in cells is still not clear. It has been demonstrated that cyclosporin A binds to an intracellular protein called cyclophilin A, a peptidyl-prolyl *cis-trans* isomerase, and inhibits its enzymic activity (Fischer et al., 1989). It was at first assumed that inhibition of this isomerase could interfere with the folding patterns of some proteins in some signal transduction pathways, or the transcription of genes involved in cytokine production. After cyclophilin was found present in all cells and tissues examined (Harding et al., 1986), the role of cyclophilin in immunosuppression was questioned. Recent experiments demonstrated that the complex of cyclophilin and cyclosporin A, as well as the complex of FK506, another immunosuppressant, and its receptor, bind to calcineurin, which is a calcium/calmodulin-activated protein phosphatase. Work with yeast has suggested that calcineurin might control the cell cycle (Reviewed by McKeon, 1991). However, we could not demonstrate the effect of cyclosporin A on proliferation of A1.1

cells. So far it is not clear whether the binding of cyclosporin to cyclophilin is a crucial target for immunosuppression and for inhibition of cell death. Nor it is clear whether the immunosuppressive effects and the interference with cell death take place through the same pathway. A cyclosporin A analogue, 6-methylalanine cyclosporine, binds strongly to cyclophilin but is not immunosuppressive (an observation cited by Halloran and Madrenas, 1991). It will be interesting to test the effect of this analogue on activation-induced cell death, as the peptidyl-prolyl *cis-trans* isomerase activity may be important in regulating cell viability. As we discussed in a later section, we believe the protooncogene *c-myc* to be possibly involved in cell death. Kronke et al. (1984) have demonstrated that cyclosporin A could indeed downregulate the expression of *c-myc*.. Thus, the mechanism of cyclosporin A-inhibited cell death also might be somehow related to the regulation of *c-myc* .

Whatever the mechanism involved, it is clear that biochemical pathways inhibited by cyclosporin A are directly implicated in activation-induced cell death. Owing to the better selectivity of cyclosporin A versus actinomycin D and cyclohexymide, the effects of cyclosporin A on activation-induced cell death both *in vitro* and *in vivo* could provide important clues to the mechanism of this phenomenon.

TGF-βs are a group of closely related peptides with a high degree of evolutionary conservation (Barnard et al., 1990). They show widespread expression in embryonic and adult tissues and essentially all cells may produce one or other isoforms of TGF-β. It is likely that nearly all cells in the body express receptors for TGF-β (Barnard et al., 1990). Therefore, TGF-β could regulate almost every cell of the mammalian organism. Depending upon particular cell types, cellular context and developmental stages, TGF-βs may act as a signal at certain times to enhance cellular proliferation, at others to inhibit cell proliferation, and at still other times to regulate cell activities related to cell differentiation. It has recently been demonstrated that TGF-β induces cell death in prostate (Martikainen and Isaacs, 1990) and cultured uterus cells (Rotello el al., 1991). In our work, we have demonstrated that TGF-β can prevent activation-induced cell death in the T cell hybridoma (Fig. VIII.6 & 7). Thus, TGF-β can have different effects on apoptosis, depending on the system involved.

The effects of TGF on immune functions are mainly suppressive. Growth of both T and B cells is suppressed by TGF-β, as is the production of antibodies by B cells, lymphokine production by T cells and the cytotoxicity of NK cells (Rook et al., 1986). TGF-βs are thus considered a natural immunosuppressant. In fact, highly immunogenic fibrosarcoma cell lines transfected with TGF-β1 gene had greater tumorogeneity, and *in vivo* administration of TGF-β2 could prevent the rejection of allografts in the mouse (Tomasi et al., FASEB presentation, 1991). TGF-βs thus shows a similar spectrum of immunosuppressive function as cyclosporin A.

TGF-βs inhibit the growth of almost all nonneoplastic epithelia in culture. However, such an inhibitory effect of TGF-β on DNA synthesis of cultured cells occurs long before the early events induced by growth factors. Recent studies have indicated that the growth-inhibitory effect of TGF-β is due to a reduction of *c-myc* expression. TGF-β could rapidly reduce c-myc mRNA and protein expression (Coffey et al, 1988). Antisense oligonucleotides corresponding to *c-myc* inhibit the growth of keratinocytes as effectively as TGF-β1 (Pietenpol et al, 1990), providing evidence that *c-myc* expression is required for keratinocyte proliferation. More directly, they found that TGF-β could down-regulate the expression of *c-myc*. Deletion analysis showed that the *cis*-acting regulatory element of *c-myc* is responsible for the suppression of TGF-β. Thus, *c-myc* downregulation might be a target effect of TGF-β1.

It has been shown that mouse amniotic fluid (MAF) from early pregnancy contains TGFβ-2, which is associated with α-fetal protein as a stable form (Altman et al., 1990). We have shown that the MAF could prevent activation-induced cell death, which could be neutralized by anti-TGFβ-2 antibody (VIII.8). When MAF from early pregnancy was examined *in vivo*, it prevented activation-induced cell death in the thymus (Fig. VIII.9). This observation might be an explanation for the interesting phenomenon of reduced cell death and increased cellularity in the mouse thymus during early pregnancy (Clarke, 1988; Clarke and Kendall, 1989).

H. Possible Involvement of *c-myc* in Activation-induced Cell Death

Apoptosis is an active process, which can be blocked by inhibition of RNA or protein synthesis. Such observations lead to the proposition that apoptosis involves activation or up-regulation of death genes. Research in this area has only recently shown some progress. There is evidence that transglutaminase (Fesus et al., 1987), SGP-2/TRMP-2 (Bursch et al., 1990) and other genes (Owens et al., 1991) are activated upon the induction of apoptosis. On the other hand, McConkey et al. (1990) have observed that a brief exposure to an RNA synthesis inhibitor led to rapid loss of endogenous endonuclease activity believed to be required for apoptosis. This indicates that inhibition of such proteins could control apoptosis. The identification of the genes involved in apoptosis could provide a key to understanding and controlling this important biological phenomenon. Due to the complexity of this process and lack of proper investigation systems little is known about these genes at present. In our experiments, we employed antisense oligodeoxynucleotides, which have been shown to offer a very successful approach to analyzing gene function especially in tissue culture (Goodchild, 1989). The basis of this approach is that antisense DNA sequences complementarily bind to specific mRNA in the cell and effectively inhibit gene function, either by inhibiting translation or by increasing RNase H-dependent duplex degradation (Stein and Cohen, 1989). Synthetic antisense oligodeoxynucleotides are added to tissue culture, where they are taken up by the cells within a short period, possibly through an 80 kd DNA binding protein on the cell membrane (Stein and Cohen, 1989). We tested several antisense oligodeoxynucleotides corresponding to protooncogenes and found that antisense oligonucleotides corresponding to *c-myc* inhibited anti-CD3-induced cell death and DNA fragmentation (Fig IX.1 & 2), but had no effect on anti-CD3-induced lymphokine production, in T cell hybridomas (Fig. IX.5). The effect of antisense to other protooncogenes was not further tested, thus we cannot make any conclusion about their effect in activation-induced cell death.

One problem with the use of conventional oligodeoxynucleotides is their rapid degradation by nucleases present in the fetal calf serum in culture media and within cells. For this reason, we also employed sulfur-derivatized oligonucleotides (Stein and Cohen, 1989) which in our studies required only 5% of the amount of conventional oligodeoxynucleotides needed for complete inhibition of cell death. These phosphorothioate oligodeoxynucleotides are DNase resistant and do not compete for ^{3}H-thymidine uptake in the assessment of DNA synthesis, required for IL-2 assays with CTL.L cells.

The product of the *c-myc* protooncogene is a highly conserved nuclear phosphoprotein whose function is related to cell proliferation and differentiation. It has been shown that constitutive expression of *c-myc* allows growth-factor dependent cells to grow in the absence of growth factor (Eilers et al., 1991), or provide a step toward malignancy (Spencer and Groudine, 1991). At the molecular level, *c-myc* protein possesses a number of conserved specific DNA binding motifs such as leucine zipper and the basic-helix-loop-helix and has been hypothesized to act directly as a transcriptional activator (Kaddurah-Daouk et al., 1987), to stimulate DNA replication (Iguchi-Aeigo et al., 1987) or to be a component involved in RNA processing (Prendergast and Cole, 1989). However, the precise genes that *c-myc* regulates and the resulting biological functions still remain largely undefined. The *c-myc* gene is one of the genes that are rapidly induced in quiescent cells upon mitogenic stimulation. The increase in *c-myc* gene transcription can be observed within a few hours after mitogenic stimulation (Reed et al., 1986). The half-life of both *c-myc* mRNA and protein is only 20-30 minutes (Moore et al., 1987; Waters et al., 1991), which makes a excellent system for its inhibition by antisense. Heikkila et al. (1987) found specific inhibition of *c-myc* expression and proliferation of human T cells when *c-myc* antisense was added 4 h before mitogenic stimulation. Holt et al., (1988) showed that antisense oligo nucleotides corresponding to c-myc induced differentiation of HL60 cells. It has also been demonstrated that the *c-myc* antisense could inhibit the growth of IL-2 dependent T cells (Harel-Bellan et al., 1988). However, *c-myc* antisense did not affect IL-2 receptor and transferrin receptor expression (Heikkila et al., 1987). This is consistent with our result of the production of IL-2 by activated T cell hybridomas in the presence of *c-myc* antisense (Fig. IX.5). These data suggest that in absence of *c-myc* cells can still express G1 phase proteins (Reed et al., 1986), but can not proliferate. Antisense oligodeoxynucleotides corresponding to *c-myc* also inhibit the growth of EBV-transformed B cells (McManaway, et al., 1990).

Our finding of the possible role of *c-myc* in activation-induced cell death adds one more biological function to this important molecule. A recent report showed that constitutive expression of *c-myc* in fibroblasts induced apoptosis when combined with cell cycle block (Evan et al., 1992). Similarly, Askew et al. (1991) demonstrated that constitutive *c-myc* expression in an IL-3 dependent bone marrow cell line accelerated apoptosis. An elevated level of apoptosis has also been shown to been specifically associated with human *c-myc* expression in a rodent fibroblast tumor (Wyllie et al., 1987). During involution of xenografts of breast carcinoma (Kyprianou et al., 1991) and prostate tissue (Quarmby et al., 1987) caused by hormone ablation, c-myc is the only major oncogene constantly raised during the period of cell death. A more relevant study to our finding is that the activation of immature thymocytes cause induction of *c-myc* (Riegel et al., 1990), as activation of these cells induces apoptosis (Chapter III and Smith et al., 1989). Therefore it is sensible to propose that c-myc is an important component of apoptosis pathway in many circumstances. This can be tested with those genes known to be expressed associated with apoptosis.

I. General Conclusion

After expressing the antigen receptor, immature T cells undergo two important selection processes in the thymus. Positive selection ensures the development of T cells with the capacity to recognize self-MHC molecules, while negative selection eliminates those with the specificity to self-antigen associated with self-MHC. In the last few years negative selection has been demonstrated in many circumstances and considered to be an important developmental process in immunological unresponsiveness to self antigens. However, the mechanism of negative selection is not known. In this thesis, we have examined the hypothesis that negative selection proceeds through activation-induced apoptosis at an immature stage during the development in the thymus. We have predicted that treatments which activate a large proportion of T cells should also induce apoptosis in a large proportion of developing thymocytes which are susceptible to negative selection.

We have demonstrated that *in vivo* injection of anti-CD3 and anti-TCRβ antibodies, which mimic the activity of antigen associated with MHC and provide polyclonal activation, is capable of inducing immature thymocytes to undergo cell death. Thus activation-induced cell death is characterized by genomic DNA fragmentation, nuclear chromatin condensation and membrane blebbing, and is restricted to the cortex, where immature thymocytes reside. It is important to note that even though injected antibodies were present in the thymic medulla, lymph nodes and the spleen, apoptosis was not observed in these tissues. Thymocyte subset analysis has confirmed that the depleted cell phenotypes are likely to be the target of negative selection described by others (Fowlkes et al., 1988; Pullen et al., 1989; von Boehmer et al., 1989). Therefore, activation of immature thymocytes results in apoptosis. The relationship between this activation-induced cell death and negative selection was supported by the observation that the potent immunosuppressive drug, cyclosporin A, which interferes with negative selection during T cell development and induces autoimmunity upon withdrawal, blocks activation-induced apoptosis in immature thymocytes. These studies strongly suggest that activation-induced apoptosis at immature stages is the mechanism of negative selection.

In order to elucidate the mechanism of negative selection, we have sought an *in vitro* model system. Murine thymocytes, however, were too unstable in culture to be useful in this regard. We have found that T cell hybridomas undergo characteristic apoptosis upon activation via the T cell receptor. Using this system, it was found that apoptosis occurs upon activation by specific antigen, mitogens, or antibodies to the T cell receptor complex. By using a nuclease inhibitor, ATA, and careful kinetic studies, it was demonstrated that DNA fragmentation is a prerequisite for activation-induced apoptosis. Apoptosis in T cell hybridomas is not observed when protein or RNA synthesis is inhibited, nor in the presence of cyclosporin A. These observations lead us to believe that certain genes must be involved. With antisense oligonucleotides, we showed that *c-myc* is a necessary component of the pathway leading to apoptosis.

Our conclusion that negative selection proceeds through the process of apoptosis has recently been challenged by Sentman et al. (1991). They demonstrated that transgenic mice bearing *bcl-2,* a mitochondrial membrane protein capable of delaying apoptosis (McDonnell et al, 1990; Strasser et al., 1991; Alnemri et al., 1992), displayed normal negative selection to endogenous superantigens. However, thymocytes from these mice are resistant to apoptosis induced by glucocorticoid, radiation, and anti-CD3 treatment. They concluded that activation-induced apoptosis might not be the experimental equivalent of negative selection. It has been shown that *bcl-2* transfection potentiates short term survival of growth factor-dependent B cell lymphoma cell lines in absence of growth factors, but *bcl-2* transfection does not potentiate long term survival (Nunez et al.., 1990). Thus, we suggest that the *bcl-2* transgene can only protect thymocytes from apoptosis induced by glucocorticoid, radiation and anti-CD3 treatment when examined at relative short terms after treatment, whereas negative selection is a persistent process and is not protected. This hypothesis is supported by Strasser et al. (1991), who have shown that in *bcl-2* transgenic Mls-2a$^+$ mice there is a complete deletion of Vβ3$^+$ cells from their periphery. However, the number of Vβ3$^+$ cells in the thymus is almost the same as in Mls-2a$^-$ mice. On the other hand, the *bcl-2* transgenic mice of Sentman et al. (1991) also have more CD3^{hi}/TCR^{hi} thymocytes than control animals. Our hypothesis of the time-dependent effect of *bcl-2* transgene can be further tested by kinetic studies on activation-induced apoptosis in the thymus of *bcl-2* transgenic mice by antigens or CD3-TCR specific antibodies. Furthermore, it is also conceivable that activation-induced apoptosis may be only one of the different mechanisms for negative selection.

Activation of mature T cells normally results in cell proliferation, and we have demonstrated that activation of immature thymocytes and T cell hybridomas results in apoptosis. Interestingly, recent experiments demonstrate that mature T cells could also be rendered susceptible to activation-induced cell death by several protocols (Newell et al., 1990; Russell et al., 1991; Lenardo, 1991), and this has been proposed to be important in maintaining peripheral tolerance. This intriguing disparity between activation-induced proliferation and cell death is probably due to different signal transduction patterns upon TCR occupancy, however, little is understood about how signal transduction leads to apoptosis. Based on the observations presented and reviewed in

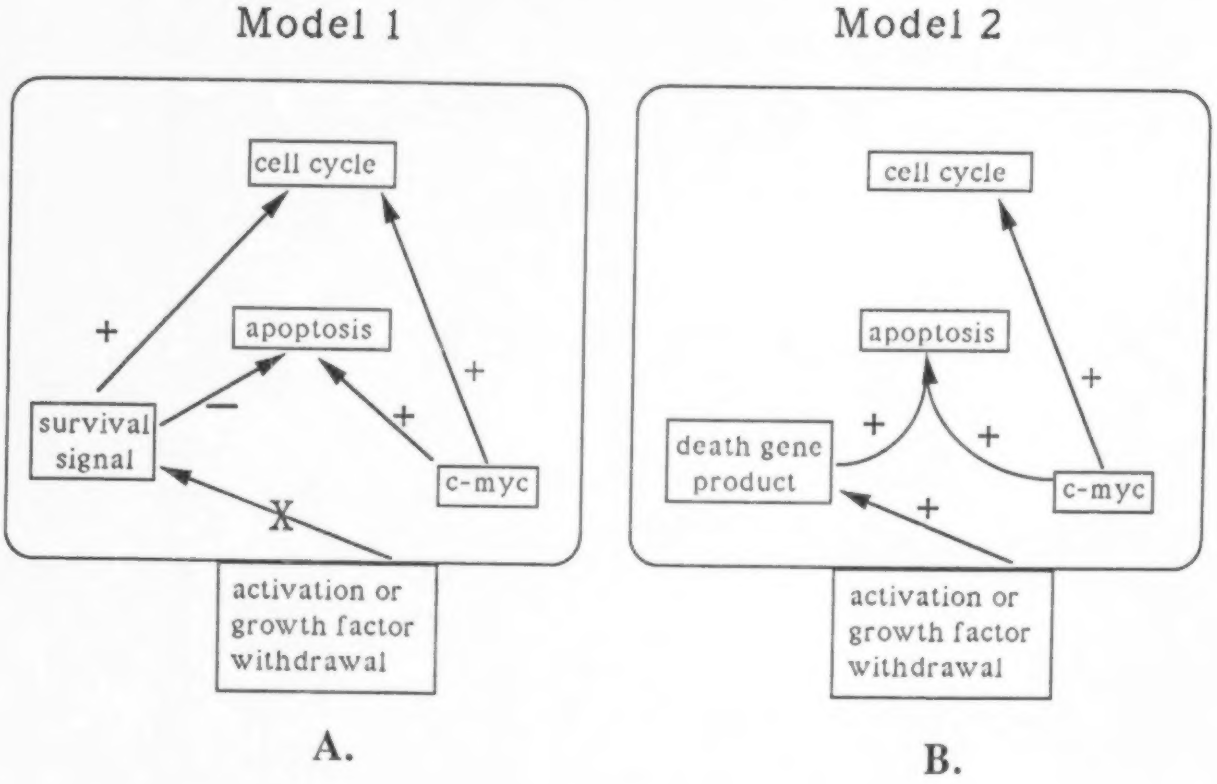

Model 1
cell cycle
apoptosis
survival signal
c-myc
activation or growth factor withdrawal
+
−
+
+
X
A.
Model 2
cell cycle
apoptosis
death gene product
c-myc
activation or growth factor withdrawal
+
+
+
+
B.

Fig. X.1. Hypothetical models of activation-induced cell death. A: The survival signal model. B: The death gene model. Detailed descriptions of the two models are presneted in the text, page 165-166.

this thesis, we propose the following two models to explain activation-induced apoptosis (Fig. X.1).

The first model is called the survival model. It proposes that cells that are susceptible to activation-induced cell death constitutively express labile proteins, such as *c-myc* or similar gene products, that are capable of promoting both cell cycle and apoptosis. When a survival signal is present, cell proliferation is ensured and apoptosis is prevented. The survival signal might be the stimulation by growth factors in the media, autocrine growth factors, or inherent intracellular signals. However, growth factor withdrawal or cell surface receptor occupancy could lead to interruption of the survival signal, and thus allow *c-myc* to drive cells to apoptosis. We can assume that the interruption process could depend on CsA sensitive components, such as calcineurin, because CsA could inhibit activation-induced cell death. The short half life of *c-myc* or of similar products could account for the observations that activation-induced apoptosis requires RNA and protein synthesis. It also explains why inhibition of *c-myc* production by antisense oligonucleotides blocks apoptosis, but not the events such as IL-2 production by signalling through the surface receptor. Activation-induced apoptosis in this model relies on the interruption of the survival signals. Such interruption can be the result of the activation of already existing kinases or phosphatases. The *bcl-2* product can be one of the candidate survival signals. It would be interesting to examine the effects of *bcl-2* on activation-induced cell death in our T cell hybridoma system and the interruption of bcl-2 function in activation of mature T cells. It is interesting to observe that in tumorigenic conversion of primary embryo fibroblasts requires the cooperation of both *myc* and *ras* (Land et al., 1983). Therefore, we propose that ras or its associated molecules could be another candidate for the survival signals in high myc expressing cells. In accordance with this proposal, it has been shown that the expression of ras increases cell survival to ionizing irradiation (Sklar, 1988). Though the system is different from activation-induced cell death, these data do provide us with interesting information.

In the above model, the proposal that *c-myc*'s short half life account for the requirement of RNA and protein synthesis by activation-induced cell death may not be accurate. Thus we propose a second model, the "death gene" model. It hypothesizes that activation through the cell surface receptor activates a number of genes, including IL-2 and TNF as well as the death gene. The death gene in this model can also be activated by growth factor withdrawal. The death gene product is restricted to apoptosis induction and not present in normal cell cycle. This model also proposes that in order to induce apoptosis, the death gene product requires *c-myc*, which drive normal cell cycle in the absence of the death gene product. At present, we do not have much support for this model, although two "death associated" genes have been cloned from glucocorticoid induced immature thymocytes (Owens et al., 1991). These two genes may not be associated with activation-induced apoptosis, because glucocorticoid and activation are antagonistic in induction of apoptosis. The identification of molecules called fragmentins from NK cells by L. Shi et al. (1992) may also shed some light on our understanding of the mechanism of activation-induced apoptosis. Recent experiments have demonstrated that these molecules are capable of inducing apoptotic type of cell death in T cell hybridomas upon introduction into these cells (Shi, L., Greenberg, A.H., unpublished observation). It would be imperative to find out if fragmentin-like proteins are induced in activation-induced apoptosis, and if they require *c-myc* to function.

In this thesis we have shown that the ligation of the T cell receptor of immature T cells leads to apoptosis and suggested this as a mechanism of self-nonself discrimination in the T cell receptor repertoire. The availability of in vitro model systems in which cells can be stimulated to undergo apoptosis in physiologically relevant ways will allow further dissection of the cellular and molecular mechanisms of apoptosis in the developing immune system.

J. Future Prospects

We can only hypothesize where future research on T cell development and apoptosis will take us in the next few years. Many unforeseen exciting discoveries have been made in these areas in the past few years, and there is no reason to suppose that they will not continue. Recent advances in these areas include an appreciation of the important role played by negative selection in maintaining self-tolerance, and of the involvement of apoptosis in many physiological and pathological processes. Further knowledge in these areas should have important clinical applications.

Negative selection of autoreactive T cells during T cell development has been very well established as a mechanism of self-tolerance. We have shown in our work that *in vivo* non-specific activation of immature T cells led to apoptosis. This result and that obtained with the *in vitro* model with T cell hybridomas hold promise for further studies. They can not only yield information on negative selection during T cell development but also provide excellent models for understanding the mechanism of apoptosis.

It is established in this thesis that activation of immature T cells leads to cell death. This disparity of response between mature and immature T cells needs to be further characterized to understand why such dramatically different outcomes result from occupancy of the same T cell receptor.

In order to establish the relationship between negative selection and avoidance of autoimmune disorders, it will be useful to find reagents which interfere with activation-induced cell death in the thymus. These reagents would then be tested for their ability to allow autoreactive T cells to escape from negative selection, leading ultimately to autoimmune diseases. Such model systems will undoubtedly provide information about autoimmunity, one of the most intriguing areas in immunology.

DNA fragmentation is the hallmark of apoptosis. We have demonstrated that DNA fragmentation plays a key role in activation-induced cell death. A major area requiring investigation is that of the mechanism leading to DNA fragmentation, which is clearly implicated in many physiological and pathological processes.

Apoptosis is an active process, which might involve the production of many products. We have demonstrated that *c-myc* is required for activation-induced cell death in T cell hybridomas. However, it is still not known how *c-myc* is involved, what regulates *c-myc* and what *c-myc* acts on. It is likely that many other genes also play key roles in the process. Further studies with activation-induced cell death in the homogeneous T cell hybridomas will help us define the genes involved in apoptosis more clearly. Further research will clearly involve a wide range of molecular and cellular studies, to provide information on the mechanisms of apoptosis. Considering the variety of discoveries made in the entire field of apoptosis in the last few years, we can confidently predict that the future holds great promise and excitement for our understanding of the role of apoptosis, especially activation-induced cell death in the immune system.

XI. REFERENCES

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